Hypertension-related, calcium-regulated gene (HCAcRG), recognized recently as COMM domain-containing 5 (COMMD5), was identified in our laboratory as a gene regulated negatively by extracellular calcium concentrations and coding for a small intracellular protein of 225 amino acids.1,2 HCAcRG is highly expressed in the kidneys and mainly located in cortical tubules. HCAcRG overexpression in cultured cells diminishes cell proliferation and DNA synthesis, causing an increase in cell size, G2/M cycle arrest, and the appearance of features of differentiated cells such as desmosome-like cell junctions.3 Besides its effect on cell proliferation and differentiation, we also demonstrated that HCAcRG overexpression augmented motility and completed wound healing 24 hours faster than control cells.4

Acute renal failure (ARF), characterized by sudden renal dysfunction resulting in impaired renal excretion of nitrogenous waste, such as blood urea nitrogen (BUN) and creatinine (Cr), is a common clinical problem with increasing incidence and mortality, poor prognosis, and unsatisfactory therapeutic options.5–8 ARF occurs in various clinical settings, including renal ischemia arising from septic shock and major cardiovascular surgery, acute drug, or toxin exposure. Ischemia/reperfusion injury (IRI) causes acute tubular injury, one of the most common forms of human ARF. We reported
Figure 1. Exogenous human HCaRG is specifically induced in renal proximal tubules (RPTs) of transgenic (Tg) mice. (A) Schematic map of the KAP2-hHCaRG construct. The KAP2 plasmid was produced by modifying the KAP-hAGT construct to replace the most of hAGT’s exon II by a NotI insertion site. The hHCaRG gene was inserted at the NotI site into the KAP2 plasmid. The transgene was excised as the SpeI and NdeI fragment for microinjection. (B) Real-time PCR analysis of hHCaRG and mHCaRG mRNA in non-Tg and Tg mice. In each experiment, expression levels were normalized to the 18S ribosomal RNA expression. hHCaRG was expressed only in Tg mice and much more in the kidneys (* \( P < 0.001 \)). mHCaRG did not differ between non-Tg and Tg mice. (C) Western blot analysis of total (mouse and human) HCaRG protein in the kidneys of non-Tg and Tg mice. Total HCaRG protein was higher in Tg mice than in non-Tg mice, * \( P < 0.05 \). (D) hHCaRG, aquaporin-1 (AQP1), KAP, and nephrin mRNA levels in tubular and glomerular fractions of renal cortex of Tg mice. hHCaRG and RPT markers were detected in the tubular enriched fraction. (E) Immunohistochemistry of total HCaRG in renal cortex of non-Tg and Tg mice. Negative control was incubated with secondary Ab only. HCaRG staining was the highest in RPTs of Tg mice. Scale bars, 50 \( \mu m \).
previously that HCaRG decreased soon after IRI in rats and reached its lowest levels at 3 and 6 hours. It then increased steadily to higher than baseline at 48 hours. Thus, HCaRG levels decline rapidly during dedifferentiation of injured proximal tubular epithelial cells (PTECs) and rise during the redifferentiation to normal functional PTECs. In this study, we generated transgenic (Tg) mice overexpressing human HCaRG (hHCaRG) in the kidneys using the kidney androgen-regulated protein (KAP) promoter, to elucidate the role of HCaRG in renal repair and regeneration. KAP was identified as an abundant renal protein under androgen control and expressed in PTECs of the renal cortex. Ding et al.11 were the first to develop Tg mice expressing human androgenotesinogen (hAGT) in the kidneys using the KAP promoter. These mice expressed endogenous and exogenous AGT and exhibited marked BP elevation. In KAP-luciferase Tg mice, luciferase expression was shown to be limited to the kidneys and reproductive tissues under androgen control. The KAP promoter allows transgene enrichment to the kidneys under androgen control.

We report here the first Tg mice with hHCaRG overexpression in the kidneys induced by the KAP promoter. These Tg mice are more resistant to ARF than negative littermate control (non-Tg) mice. After IRI, which produced similar damage in non-Tg and Tg mice, we observed more rapid inhibition of proliferation and acceleration of PTEC redifferentiation via p53-independent p21 transactivation, and less inflammation, culminating in more rapid recovery of renal function and better survival of Tg mice.

**RESULTS**

**Generation and Analysis of hHCaRG Tg Mice**

Tg mice were generated to produce human HCaRG in renal proximal tubules (RPTs) by inserting the open reading frame of hHCaRG under control of the KAP promoter (Figure 1A) as described previously.13,14 In male Tg mice, hHCaRG mRNA expression was increased in adults under androgen control. At baseline, the phenotypes of non-Tg and Tg mice were comparable with regard to body weight, BP, and glomerular size, as well as renal function (Supplementary Table 1). HCaRG mRNA was mainly expressed in the kidneys, followed by the brain, with low expression in the heart or other organs examined and no expression in non-Tg mice (Figure 1B). Endogenous mouse HCaRG (mHCaRG) was not affected by the transgene, and its expression levels did not differ between non-Tg and Tg mice in all organs. As shown in Figure 1C, total HCaRG protein expression was more than three-fold higher in the kidneys of Tg mice compared with non-Tg mice.

To precisely localize the expression of hHCaRG in the kidney, the tubular and glomerular fractions of the renal cortex of Tg mice were separated by differential sieving. Figure 1D shows that hHCaRG is coexpressed with two RPT markers, aquaporin-1 (AQP1)15 and KAP, and not with the glomerular marker nephrin. This was confirmed by immunohistochemistry using anti-HCaRG antibody (Ab). The kidney from Tg mice showed higher staining of total HCaRG principally in RPTs compared with non-Tg mice (Figure 1E). No staining was detected in glomeruli. These results indicate that hHCaRG is specifically induced in the RPTs of Tg mice.

**Recovery after ARF in hHCaRG Tg Mice**

To induce ARF, we produced an IRI model in mice. Fifty minutes of ischemia and contralateral nephrectomy caused severe tubular damage that resulted in pronounced renal dysfunction in both non-Tg and Tg mice. The survival rates of non-Tg and Tg mice were compared after IRI (Figure 2A). Mortality occurred between 24 and 72 hours after IRI in both non-Tg and Tg mice. After 2 days, the percent survival rates were 86 and 56% for Tg mice and non-Tg mice, respectively. The survival rate 7 days after IRI was 64% in Tg mice compared with only 25% in non-Tg mice (P = 0.0249). In addition, Tg mice renal function, as determined by plasma BUN and Cr, was improved significantly faster in comparison with non-Tg mice after 2 days (Figure 2B). To confirm that ARF was present and to investigate the effects of HCaRG overexpression on tubular damage, we next assessed the renal morphologic changes (Figure 2C). The postischemic kidneys of non-Tg mice displayed severe tubular damage. Although there were no significant differences between non-Tg and Tg mice at day 1 (Supplementary Figure 1), the severity score of most of the parameters evaluated was significantly lower in Tg mice at day 2. The expression profile of hHCaRG and AQP1 after IRI is shown in Figure 2D. AQP1 was markedly reduced at day 1, irrespective of the mouse genotype and then recovered more in Tg than non-Tg mice after 7 days. The hHCaRG expression profile followed that of AQP1, suggesting that the reduction of hHCaRG after IRI was due to RPT loss. Taken together, these results indicate that HCaRG overexpression improved the recovery from acute tubular damage and renal dysfunction and reduced mortality in Tg mice.

**Cell Proliferation and Apoptosis after IRI**

Figure 3A reports the changes in kidney weight after IRI or nephrectomy. As expected, nephrectomy elicited moderate compensatory renal hypertrophy, with no differences between non-Tg and Tg mice. IRI increased kidney weight, which was significantly lower in Tg mice after 2 days. Such inhibition of the increase in kidney weight by HCaRG could have resulted from the stimulation of cell death or inhibition of cell growth. We therefore examined differences in cell apoptosis and proliferation. Apoptotic cell death, as detected by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) procedure in postischemic kidneys of both non-Tg and Tg mice, was significantly increased and peaked at day 1, but HCaRG overexpression had no effect on apoptosis (Figure 3B). At day 2, HCaRG overexpression significantly suppressed the increase of proliferating cell nuclear antigen (PCNA) expression in the kidneys after IRI compared with non-Tg mice (Figure 3, C and D). To detect proliferating cells in tubules and
Figure 2. hHCaRG transgenic (Tg) mice are resistant to ischemia/reperfusion injury (IRI). (A) Survival curves of non-Tg mice (black line) and Tg mice (red line) after IRI (n = 14 to 16). The survival rate 7 days after IRI in Tg mice was significantly (P = 0.0249) higher than that of non-Tg mice. (B) Changes in serum creatinine (Cr) and blood urea nitrogen (BUN) levels. Renal function in Tg mice improved more rapidly in Tg mice than non-Tg mice. *P < 0.01 compared with non-Tg nephrectomized mice. †P < 0.05 compared with non-Tg IRI mice. (C) Morphologic damage scores after ARF at day 2. Morphologic findings were scored according to cast deposition, tubular dilation (Dilation), tubular degeneration (Degeneration), tubular necrosis (Necrosis), and proximal tubular brush-border loss (BB loss). The severity score of tubular damages was significantly lower in Tg mice, *P < 0.05. Scale bars, 100 μm. (D) hHCaRG and aquaporin-1 (AQP1) expression profiles after IRI. hHCaRG showed an expression pattern similar to that of AQP1 *P < 0.05 compared with AQP1 in non-Tg mice.
Figure 3. hHCaRG transgenic (Tg) mice showed lower tubular cell proliferation after ischemia/reperfusion injury (IRI). (A) Changes in kidney weight after IRI. IRI increased kidney weight, which was lower in Tg mice after 2 days. *P < 0.05 compared with non-Tg nephrectomized mice. †P < 0.05 compared with non-Tg IRI mice. KW/ BW, kidney weight/body weight. (B) TUNEL staining of kidneys at day 1 after IRI. The number of TUNEL-positive cells per field was counted in transverse fields until 2 days after IRI. Apoptotic cell death was comparable between non-Tg and Tg mice. *P < 0.01 compared with non-Tg nephrectomized mice. Scale bars, 100 μm. (C) IRI increased PCNA mRNA expression in the kidneys. HCaRG overexpression suppressed PCNA expression at day 2. *P < 0.05 compared with non-Tg nephrectomized mice. †P < 0.05 compared with non-Tg IRI mice. (D) Western blot analysis of PCNA protein in the kidneys at day 2. The increase of PCNA protein was less in Tg mice. *P < 0.05. †P < 0.01 between IRI and nephrectomy. (E) Localization of proliferating cells at day 2 after IRI. The number of PCNA- and Ki-67-positive cells was counted in tubular and interstitial regions, respectively. Proliferating cells in tubules were lower in Tg mice than in non-Tg mice. The black arrow indicates PCNA- or Ki-67-positive tubular cells. The white arrow indicates PCNA- or Ki-67-positive interstitial cells. *P < 0.05. Scale bars, 50 μm.
interstitium, PCNA and Ki-67 were analyzed by immunohistochemistry in postischemic kidneys after 2 days (Figure 3E). IRI dramatically augmented the number of proliferating cells in tubules and interstitium. HCaRG overexpression diminished the number of proliferating cells in tubules significantly more in Tg mice, whereas the number of proliferating cells in the interstitium was comparable between non-Tg and Tg mice. Thus, we can conclude that the inhibition of IRI-induced increase in kidney weight by HCaRG overexpression was mainly due to inhibition of PTEC proliferation rather than by promotion of apoptosis.

**Effect of HCaRG Overexpression on Tubular Redifferentiation**

The expression levels of p53 and p21 in postischemic kidneys are depicted in Figure 4A. The expression of p53, which acts as a p21 transcriptional activator, increased at day 1 after IRI with a gradual decrease thereafter, but with no effect of the transgene. p21 was also up-regulated in postischemic kidneys and peaked at day 1. Its expression in non-Tg mice showed a pattern similar to p53, but in Tg mice, it remained high at day 2 and 3. The expression of E-cadherin, a marker of tubular epithelial integrity, was significantly decreased in postischemic kidneys of non-Tg mice at day 2, whereas in Tg mice, E-cadherin recovered to levels similar to that in the nephrectomized controls at day 3 (Figure 4B). The expression of vimentin, an intermediate filament protein that is only expressed in mesenchymal cells, was markedly elevated in postischemic kidneys at day 1, irrespective of the transgene (Figure 4B). However, vimentin induction declined much more rapidly in Tg mice after 2 days.

**Anti-inflammatory Effects of HCaRG**

As reported previously, IRI resulted in up-regulation of proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, and monocyte chemotactic protein (MCP)-1 at day 1, irrespective of HCaRG expression (Figure 5A). HCaRG overexpression rapidly reduced these inflammatory mediators compared with non-Tg mice at day 2. In addition, the infiltration of F4/80-positive macrophages in postischemic kidneys was significantly decreased in Tg mice, in accordance with a reduction of proinflammatory mediators (Figure 5B).

**Confirmation of HCaRG Effects in Cultured Cells**

Madin-Darby canine kidney (MDCK)-C7 cells were stably transfected with control plasmid (Neo-MDCK) or HCaRG expression plasmid (HCaRG-MDCK) as described previously. HCaRG-MDCK cell proliferation was lower than Neo-MDCK cells (Figure 6A). HCaRG overexpression did not protect from initial hypoxic shock until 24 hours; however, it improved cell viability after 72 hours under hypoxic conditions (Figure 6B), pointing again to an effect on cell repair rather than a protective effect against cell injury. Cell protein lysates were subjected to Western blotting to ascertain the effect of HCaRG on the expression pattern of specific proteins (Figure 6C, left panel). HCaRG was detected only in HCaRG-MDCK cells. E-cadherin levels were higher in HCaRG-MDCK cells than Neo-MDCK cells. In contrast, vimentin levels were lower in HCaRG-MDCK cells. HCaRG overexpression caused p21 upregulation, whereas p53 expression was comparable between Neo- and HCaRG-MDCK cells. We also studied the effect of short hairpin RNA (shRNA) directed against HCaRG in HCaRG-MDCK cells (Figure 6C, right panel). At the protein level, HCaRG expression was reduced by 70%, compared with a nontarget control. p21
expression was decreased in shRNA-HCaRG-treated HCaRG-MDCK cells. Moreover, the increase of E-cadherin and the reduction of vimentin in HCaRG-MDCK cells were reversed by shRNA-HCaRG treatment. Thus, HCaRG overexpression specifically induced p21 transactivation and facilitated differentiation in HCaRG-MDCK cells. The results in cell culture confirmed the expression pattern seen in kidney extracts in vivo.

**DISCUSSION**

Despite better knowledge of the pathophysiological pathways underlying kidney diseases at both the basic and clinical levels, acquired during the last decade, the progression of renal dysfunction and the number of hemodialyzed patients are still steadily increasing. ARF after IRI is the result of renal isch-
emia with renal hypoperfusion and hypoxia, leading to PTEC damage, physiologic dysfunction, and death. Restoration of blood flow by reperfusion injures endothelial cells and then activates a number of inflammatory mediators. IRI models are widely investigated to elucidate etiological mechanisms and to identify factors that are involved in repair and regeneration for the treatment of ARF. The kidneys have the ability to recover from renal damage and dysfunction, unlike the heart and the brain. Repair of RPTs requires dedifferentiation, proliferation, and migration of surviving PTECs to replace dead cells after ARF. Dedifferentiated PTECs acquire a rapidly proliferating and highly-migrating phenotype in the early stage of renal repair processes.

In studies of mice ARF models, ischemia for 40 to 60 minutes generally produces between 60 and 80% mortality. In these experiments, IRI was induced by left pedicle clamping for 50 minutes, followed by contralateral nephrectomy at the time of reperfusion. Ischemia for 50 minutes led to mortality in 75% of non-Tg mice. HCaRG overexpression reduced mortality to 36% and diminished renal dysfunction and morphologic tubular damage. To determine whether this improvement of the survival rate resulted from kidney protection against IRI or the stimulation of renal repair, we analyzed the factors associated with the renal injury process (Supplementary Figure 2). Kidney injury molecule-1 is a known biomarker of RPT injury in ARF. TGF-β1 stimulates the epithelial-to-mesenchymal transition of injured PTECs by reducing cell adhesion molecules. Kidney injury molecule-1, TGF-β1, extracellular matrices, and fibroblast specific protein-1 (FSP1) were markedly increased in postischemic kidneys after 2 days and were comparable between non-Tg and Tg mice. In addition, HCaRG overexpression did not affect AQP1 levels and the severity of tubular damages at day 1 (Supplementary Figure 1). E-cadherin and vimentin levels were initially not different between non-Tg and Tg mice 1 day after IRI. These data indicate that IRI caused similar initial damage, loss of PTECs, and tubular dedifferentiation in non-Tg and Tg mice, whereas HCaRG overexpression significantly improved RPT repair and survival rate in Tg mice.

Tubular epithelial cells and cells within interstitial spaces, which are associated with vascular network components and resident fibroblasts, account for about 80% of kidney volume. As expected, IRI increased kidney weight that could be due to edema, hypertrophy and cell proliferation. HCaRG overexpression did not reduce initial edema and hypertrophy in nephrectomized controls and in the unilateral ureteral obstruction (UUO) model (Supplementary Figure 3). The increase of kidney weight by IRI was significantly diminished in Tg mice after 2 days. Although HCaRG overexpression did not affect reduced the protein expression of HCaRG by 70%, compared with a nontarget control. HCaRG suppression led to lower p21 expression and abolition of E-cadherin up-regulation and vimentin downregulation in HCaRG-MDCK cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA.

Figure 6. HCaRG inhibits cell proliferation by facilitating differentiation in cultured cells. (A) Cell proliferation in Neo- and HCaRG-Madin-Darby canine kidney (MDCK) cells. HCaRG inhibited cell proliferation in HCaRG-MDCK cells. *P < 0.01. (B) Cell viability in Neo- and HCaRG-MDCK cells under hypoxic conditions. HCaRG did not protect from initial hypoxic shock but improved cell viability in HCaRG-MDCK cells after 72 hours. *P < 0.01. (C) Protein expression of HCaRG, differentiation phenotype markers (E-cadherin and vimentin), p53, and p21 in Neo- and HCaRG-MDCK cells with or without shRNA. HCaRG was expressed only in HCaRG-MDCK cells. In HCaRG-MDCK cells, E-cadherin was up-regulated, and vimentin was down-regulated compared with Neo-MDCK cells. HCaRG induced p21 transactivation without the p53 induction. shRNA-HCaRG treatment
apoptotic cell death, it slowed down necrosis and the increment of PCNA expression 2 days after IRI, consistent with the lower increase of kidney weight. IRI is known to cause epithelial dedifferentiation, followed by features of rapidly-dividing cells under controlled growth. A large number of proliferating cells were apparent in the tubules at day 2. HCaRG overexpression decreased only the number of proliferating tubular cells. The number of proliferating fibroblasts in the interstitium was comparable between non-Tg and Tg mice. HCaRG overexpression did not affect initial edema but reduced the increase of kidney weight by controlling PTEC proliferation after ARF, with no effect on fibroblast proliferation.

p21 has been demonstrated to be induced in cells undergoing either p53-associated G1 arrest or apoptosis but not in cells arrested in G1 or resulting in apoptosis through p53-independent mechanisms (Figure 7A). In this study, the number of apoptotic cell nuclei peaked at day 1 and then decreased with no difference in cell death between non-Tg and Tg mice (Figure 3B). p53 and p21 mRNA expression levels were also higher than in control mice at day 1, followed by a gradual decline in their expression in both non-Tg and Tg mice. However, after 2 days, p21 mRNA expression remained elevated only in Tg mice independently of p53 expression and apoptosis (Figure 4A). These data suggest that the p53 and p21 peaks at day 1 are mainly associated with the induction of apoptosis and that HCaRG overexpression induces p21 transactivation via a p53-independent pathway in the recovery phase after injury. This increase of p21 could control cell proliferation rather than apoptosis.

According to previous reports, p21 transactivation by a p53-independent pathway also occurs in renal tubules, but not in the glomerulus or interstitium during the recovery phase after ARF. In p21 knockout mice, ARF causes more rapid onset of renal dysfunction and induces more severe morphologic damage with a three-fold higher mortality rate than in normal mice. Miyaji et al. reported that cisplatin-induced ARF led to two peaks of increased p21. The first peak was accompanied by up-regulation of p53 and PCNA, possibly reflecting G1 arrest and DNA repair. The second p21 peak occurred through a p53-independent pathway and might have contributed to cell differentiation. These studies indicate that p53-independent p21 up-regulation could be crucial in controlling epithelial proliferation and morphogenesis in the kidneys. We previously observed that HCaRG-overexpressing HEK293 cells exhibit features of epithelial cell differentiation at ultrastructural levels, including the presence of desmosome-like junctions, microvilli, and other features of junctional complexes that are correlated with p21 up-regulation. In postischemic kidneys, the decrease of E-cadherin recovered more rapidly in Tg mice, and vimentin was not visible in Tg mice 2 days after IRI. Furthermore, E-cadherin was also increased, and vimentin was lower through p21 induction without p53 up-regulation in HCaRG-MDCK cells. The proliferation of HCaRG-MDCK cells was reduced, and cell viability under hypoxic conditions was improved after 3 days, because HCaRG-MDCK cells demonstrated a more mature epithelial phenotype than Neo-MDCK cells. In addition, these effects were reversed by HCaRG inhibition with shRNA-HCaRG. Taken together, these findings suggest that HCaRG improves the survival of resident PTECs by accelerating redifferentiation via p21 transactivation through its p53-independent pathway during the regeneration phase after ARF.

Inflammatory signaling cascades, which are initiated by endothelial dysfunction, are augmented dramatically in ischemic RPTs by the generation of numerous inflammatory mediators. Macrophages accumulate and infiltrate the renal parenchyma in response to IL-1β and MCP-1 up-regulation by

Figure 7. HCaRG accelerates tubular repair after ARF. (A) ARF mainly damages PTECs and causes cell death as a result of necrosis and apoptosis. The induction of cell death is associated with p21 up-regulation dependent on the p53 pathway. Sublethally-injured PTECs dedifferentiate to mesenchymal cells, and then macrophages infiltrate in the tubulo-interstitium. (B) Dedifferentiated PTECs proliferate and migrate to repair the denuded area. HCaRG promotes PTEC migration and accelerates redifferentiation of PTECs by controlling its proliferation via p21 transactivation through the p53-independent pathway and then inhibits the infiltration of macrophages.
TNF-α.41 TNF-α seems to be central to the activation of these proinflammatory chemokine and cytokine responses in ARF. We noted that IRI induced the up-regulation of inflammatory mediators and the infiltration of F4/80-positive macrophages. The inflammatory reaction at day 1 was not significantly different between non-Tg and Tg mice (Figure 5A). However, HCaRG overexpression rapidly reduced these activators of inflammatory responses 2 days after IRI. HCaRG appeared to play a role in maintenance of the epithelial cell barrier by promoting the redifferentiation of damaged PTECs, thus reducing macrophage infiltration and inflammation after ARF.

This study is the first one to report that HCaRG has a beneficial effect in postischemic kidneys during the repair phase by controlling cell proliferation, facilitating redifferentiation, and reducing inflammation. These tubule-regenerating functions and anti-inflammatory effects are the most important factors for the improvement of renal function and survival. Similar results were obtained in UUO model in hHCaRG Tg mice (Supplementary Figure 3). HCaRG overexpression controlled PTEC proliferation during later phase of UUO, without affecting initial edema or hypertrophy. In conclusion, our data support the idea that HCaRG accelerates tubular redifferentiation after ARF by promoting PTEC migration and controlling its cell cycle via p21 induction through its p53-independent signaling pathway (Figure 7B). HCaRG could thus play an important role in the repair of injured PTECs to preserve the viability, tubular function, and epithelial barrier after ARF. Furthermore, the acceleration of healing and inflammatory blockade by HCaRG could serve as a new therapeutic approach to ARF.

CONCISE METHODS

All of the procedures in this project conformed to guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Research Centre, Centre Hospitalier de l’Université de Montréal.

Generation of hHCaRG Tg Mice

hHCaRG Tg mice were generated to express hHCaRG in RPTs under the control of the KAP promoter, which is responsive to testosterone stimulation. The KAP2 plasmid was produced by modifying the KAP-hAGT construct to replace most of hAGT’s exon II by a NotI insertion site (a generous gift from Dr. Curt D. Sigmund at the University of Iowa). To generate plasmid KAP2-hHCaRG, we PCR-amplified a cDNA-encoding hHCaRG protein from human genomic DNA. The hHCaRG cDNA amplicon was inserted at the NotI site of exon II into a KAP2 plasmid containing the KAP promoter. The final KAP2-hHCaRG transgene was excised by digestion with NdeI and SpeI, purified by agarose gel electrophoresis, recovered by gel extraction, and then microinjected into one-cell fertilized mouse embryos obtained from superovulated B6/C3F1 (C57BL/6 X C3H) mice as described previously.14,42 The presence of the transgene was identified by PCR of tail genomic DNA using hHCaRG specific primer sets (5’-GGGCCAGGTCCCTCAGAGG-3’ and 5’-AAGTCAGCAA-CATGGCGGAGC-3’) as described previously.43 The PCR conditions for genotyping the hHCaRG transgene were: denaturation for 5 minutes at 95°C, then 30 cycles for 30 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 72°C, followed by 5 minutes at 72°C. hHCaRG Tg mice were backcrossed onto a C57BL/6 background for at least seven generations before renal IRI experiments.

To determine the localization of hHCaRG, tubular and glomerular enriched fractions of the renal cortex of Tg mice were separated by differential sieving. For total hHCaRG immunohistochemistry, 3-μm-thick kidney sections were deparaffinized, hydrated, and heated for antigen unmasking. After blocking with goat serum (Invitrogen, Carlsbad, CA), the sections were incubated with diluted primary Ab (Proteintech Group Inc., Chicago, IL) overnight at 4°C, washed in PBS, and incubated with biotinylated goat anti-rabbit IgG (Invitrogen) for 30 minutes. The reaction products were visualized using CTS kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s recommendations. Finally, sections were counterstained with Mayer’s hematoxylin. Negative control consisted of incubations with secondary Ab only.

Ischemia/Reperfusion

An experimental IRI model was performed in 10- to 12-week-old male hHCaRG Tg mice and non-Tg littermates. All of the mice received a subcutaneous injection of 50 μg/kg buprenorphine (Temgesic; Schering-Plough, Kenilworth, NJ) for analgesic purposes. After inducing isoflurane anesthesia, the left kidney and renal vessels were exposed via a dorsal incision. The left renal artery was clamped with a small vascular clamp for 50 minutes and released. Occlusion was verified visually by a change in kidney color, and reperfusion was verified by appearance of the original color. In addition, the right kidney was nephrectomized via a dorsal incision.26,44 The nephrectomized group without IRI induction served as the controls (n = 4 to 6/group). During surgery, body temperature was maintained by placing the mice on a 38°C heating pad. After surgery, they were returned to their cages and allowed free access to food and water. Blood was drawn at the time of sacrifice to quantify BUN and Cr concentrations in serum. The mice were sacrificed 1, 2, 3, and 7 days after IRI (n = 6 to 10/group), and their kidneys were harvested for histologic and biochemical analyses.

Histopathology

Kidneys from IRI mice were fixed in Tissuefix (Biopharm Inc., Hatfield, AR), embedded in paraffin, sectioned into 3-μm slices, and stained with hematoxylin and eosin and periodic acid–Schiff at Institut de Recherche en Immunologie et Cancérologie in Montreal. Histologic examination was undertaken by light microscopy by a pathologist blinded to the experimental groups. The percentage of morphologic damage to the kidney after IRI was estimated according to the following criteria: cast deposition, tubular dilation, tubular degeneration, tubular necrosis, and proximal tubular brush-border loss. These lesions were evaluated on a scale of 0 to 4 as: 0, not present; 1, mild; 2, moderate; 3, severe; and 4, very severe.44,45
Survival after IRI
The survival rates of non-Tg and Tg mice (n = 14 to 16/group) were compared by the Kaplan and Meier method.45 Statistical significance was assessed by the log-rank test.

mRNA Quantification and Expression
Total RNA was isolated from renal tissue with TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Isolated total RNA was reverse-transcribed as described previously.4 Real-time quantitative PCR was performed with diluted cDNA using FastStart TaqMan Probe Master and FastStart SYBR Green Master (Roche, Mannheim, Germany) in a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia), according to the manufacturer’s instructions. All assay-on-demand primers and probes were purchased from Applied Biosystems (Foster City, CA). MCP-1 and IL-1β were detected by their specific primer sets (MCP-1: 5’-TG-CATCTGCCTAAGGTCTTC-3’ and 5’-AAGTGCTTGAGGTGGTGG-3’, IL-1β: 5’ and 3’ primers: 5’-CCCTCAGGATGAGGACAATGA-3’ and 5’-AACGTCACACACACAGGGTT-3’).46 Real-time PCR data were analyzed with standard curves and normalized to 18 S ribosomal RNA with its specific primer sets (5’ and 3’ primers: 5’-CGGCTACCACATCCAGGAA-3’ and 5’-GCTGGAATTACCGCGGCT-3’) as described previously.47 Correlation coefficients for standard curves were all >0.95.

Western Blot Analyses
Kidney tissues and MDCK cells were lysed either in SDS buffer (0.3% SDS, 10 mM Tris-HCl, pH 7.4) or in a modified radioimmunoprecipitation assay protein extraction buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 50 mM Tris-HCl, pH 7.4, 1 mM Na2VO4, 1 mM NaF, 1 mM phenylmethylsulfonlfyl fluoride) with added proteases inhibitors (Roche), frozen/thawed, trituated, and centrifuged. Total proteins were mixed with sample buffer containing 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol and then heated for 5 minutes at 95°C. Each sample was applied equally to 8 to 10% polyacrylamide gels and transblotted to polyvinylidene fluoride membranes (GE Healthcare, Uppsala, Sweden). After blocking and washing, primary Ab to p53, PCNA, the glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Canvers, MA), E-cadherin, p21Cip1/WAF1 (BD Biosciences, Mississauga, Canada), vimentin (Sigma-Aldrich, St. Louis, MO), and HCaRG were included overnight, followed by incubation with secondary horseradish peroxidase-conjugated Ab (Santa Cruz Biotechnology, Inc.) for 1 hour. Immunocomplexes were detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Waltham, MA). In each experiment, expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

Quantification of Apoptosis
Apoptotic cell death was quantified with the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI). Briefly, 3-μm-thick kidney sections were deparaffinized, hydrated, and treated with protease K at 20 μg/ml in Tris-EDTA buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA) for 10 minutes. After pre-equilibration, strands of DNA were end-labeled by incubation with recombinant terminal deoxynucleo-
tidy transferase for 1 hour at 37°C. The reaction was stopped by adding 2X sodium chloride/sodium citrate hybridization buffer for 15 minutes. After washing, the slides were mounted and viewed at 200× by fluorescence microscopy. Apoptotic cells were counted in 20 randomly-chosen nonoverlapping renal cortex fields per section for each mouse and expressed as apoptotic cells per field.

Immunolocalization of Proliferating Cells
PCNA localization in the renal cortex was detected on paraffin-embedded sections according to a heat-induced epitope retrieval method following the manufacturer’s recommendations (PCNA staining kit; Invitrogen). Ki-67 immunostaining was performed on paraffin-embedded sections according to heat-induced epitope retrieval method using rat anti-mouse Ki-67 Ab (Dako, Glostrup, Denmark). The reaction products were visualized using a CTS kit in accordance with the manufacturer’s recommendations. PCNA- and Ki-67-positive cells were counted separately in 20 randomly-chosen nonoverlapping renal cortex fields (200×) of tubular or interstitial areas per section for each mouse. The results were expressed as the number of PCNA- and Ki-67-positive cells per field in tubular and interstitial regions, respectively.

Quantification of Macrophages
For macrophage assessment, sections underwent antigen retrieval with proteinase K (20 μg/ml), followed by incubation with goat se-
rum. Rat anti-mouse F4/80 Ab (Serotec, Oxford, UK), incubated for 1 hour at room temperature, served to localize macrophages. The slides were washed in PBS before incubation with goat anti-rat FITC sec-
ondary Ab (Santa Cruz Biotechnology, Inc.) for 30 minutes. The number of macrophages was counted in 20 randomly-chosen nonoverlapping renal cortex fields (400×) per section for each mouse. The results were expressed as the number of F4/80-positive cells per field.

Stable Transfection and Cell Culture
MDCK-C7 cells were transfected with control plasmid (pcDNA/Neo; Invitrogen) or plasmid encoding rat HCaRG according to the Fu-
GENE method (Roche) as described previously.4 Transfected cells were first selected with 800 μg/ml G418 (Invitrogen), and single clones were isolated. These clones were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum with 400 μg/ml G418 and 10 μg/ml penicillin/streptomycin.

Cell Proliferation and Viability Assay
Stable transfected cells or control cells were seeded onto 96-well mi-
croplate plates under normoxic conditions. For hypoxic conditions, the cells were cultured in a humidified atmosphere at 5% CO2 with less than 1% O2. Cell proliferation and viability assays were performed with cell proliferation reagent WST-1 (Roche) according to the manu-
facturer’s recommendations. WST-1 was added to the culture me-
dium at each time point, and the cells were incubated at 37°C for 30 minutes. WST-1 absorption was measured at 450 nm in a multilabel counter 1420 Victor® V (PerkinElmer Life Sciences). Cell viability was presented as percentage of viable cells relative to controls under nor-
moxic conditions.

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shRNA Knockdown
HCaRG was knocked down with shRNA expression plasmids (pSilencer 3.0-H1; Applied Biosystems) targeting a HCaRG sequence spanning from 134 to 152 (5’-GGAGCACCTTCAGAAAGTT-3’).

Statistical Analyses
The animals were randomly divided into nephrectomized control and IRI groups. The Western blotting data were analyzed by measuring the intensity of the hybridization signals with Image J from the National Institutes of Health.48 The values are reported as the means ± SEM. Unpaired data were analyzed by t test. Two-way ANOVA and Duncan’s multiple range test were also used. P < 0.05 was considered to be statistically significant.

REFERENCES


