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MAGE genes in the kidney: identification of *MAGED2* as upregulated during kidney injury and in stressed tubular cells

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ABSTRACT

Background. Mutations in *Melanoma Antigen-encoding Gene D2* (*MAGED2*) promote tubular dysfunction, suggesting that MAGE proteins may play a role in kidney pathophysiology. We have characterized the expression and regulation of MAGE genes in normal kidneys and during kidney disease.

Methods. The expression of MAGE genes and their encoded proteins was explored by systems biology multi-omics (kidney transcriptomics and proteomics) in healthy adult murine kidneys and following induction of experimental acute kidney injury (AKI) by a folic acid overdose. Changes in kidney expression during nephrotoxic AKI were validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), western blot and immunohistochemistry. Factors regulating gene expression were studied in cultured tubular cells.

Results. Five MAGE genes (*MAGED1*, *MAGED2*, *MAGED3*, *MAGEH1*, *MAGEE1*) were expressed at the mRNA level in healthy adult mouse kidneys, as assessed by RNA-Seq. Additionally, *MAGED2* was significantly upregulated during experimental AKI as assessed by array transcriptomics. Kidney proteomics also identified *MAGED2* as upregulated during AKI. The increased kidney expression of MAGED2 mRNA and protein was confirmed by qRT-PCR and western blot, respectively, in murine folic acid- and cisplatin-induced AKI. Immunohistochemistry located MAGED2 to tubular cells in experimental and human kidney injury. Tubular cell stressors

[serum deprivation and the inflammatory cytokine tumour necrosis factor-like weak inducer of apoptosis (TWEAK)] upregulated *MAGED2* in cultured tubular cells.
Conclusions. MAGED2 is upregulated in tubular cells in experimental and human kidney injury and is increased by stressors in cultured tubular cells. This points to a role of MAGED2 in tubular cell injury during kidney disease that should be dissected by carefully designed functional approaches.

Keywords: acute kidney injury, chronic kidney disease, inflammation, MAGED2, tweak

INTRODUCTION

Chronic kidney disease (CKD) affects 10% of the adult population and may progress to end-stage renal disease (ESRD) [1]. CKD is also associated with increased risk of premature mortality and of acute kidney injury (AKI), which has a mortality of around 50% and is, in turn, associated with an increased risk for CKD progression [2]. The current therapy for both AKI and CKD is unsatisfactory and CKD is among the fastest growing causes of death worldwide [1, 3].

MAGE stands for Melanoma Antigen-encoding Gene and the MAGE gene family includes 55 genes in humans and 30 in mice, which are located in various chromosomes [4]. *MAGE1* (renamed as *MAGEA1*) was the first MAGE gene to be cloned from a human melanoma cell line, and encodes the melanoma MZ2-E antigen [5]. Proteins encoded by MAGE genes share a highly conserved sequence of nearly 200 residues known as the MCD or MAGE Conserved Domain [6]. MAGE genes have been classified in two groups, according to the expression pattern [7–9]. Type I MAGE subfamily genes, such as *MAGEA*, *MAGEB* and *MAGEC*, are only expressed in cancer cells and germinal cells, while type II MAGE subfamily genes, such as *MAGED2*, are also found in healthy somatic tissues. Type I subfamily genes have no introns and are all located in subtype-specific clusters [10]. They have been studied as targets for cancer vaccine development [11]. Type II subfamily genes have more heterogeneous structures and genes are located dispersed all over the genome. This subfamily encompasses two groups according to the gene structure. MAGED (*MAGED1*, *MAGED2*, *MAGED3* and *MAGED4*) genes have 13 exons while another set of genes has 1 exon (*MAGEE1*, *MAGEE2*, *MAGEF1*, *NSMCE3*, *MAGEH1*, *MAGEL2* and *NDN*).

MAGED family genes are highly conserved among mammalians even outside of the MCD [8, 12]. *MAGED1* (NRAGE) is involved in the P75 neurotrophin receptor apoptosis pathway [13] and *MAGED1* deficiency is associated with low bone density in mice [11]. *MAGED2* has been identified as the ancestor of MAGED genes [11]. *MAGED2* is expressed widely in adult mice [6] and has been involved in neurogenesis [4] and in cell cycle regulation by binding p53 [14]. Recently, *MAGED2* came to the attention of the nephrological community when it was reported that truncating *MAGED2* mutations caused transient antenatal Bartter syndrome, which is characterized by severe polyhydramnios that may cause premature delivery [15, 16]. It was labelled 'transient' since Bartter syndrome resolved after birth. Polyhydramnios results from the imbalance between excessive foetal polyuria and removal of amniotic fluid by foetal swallowing [17]. Polyuria results from defective salt reabsorption along the thick ascending limb of the loop of Henle, which impairs the urine concentration ability [18]. In the kidney, *MAGED2* was mainly found in tubular cells of the thick ascending limb of the loop of Henle and the distal convoluted tubule. In these cells, *MAGED2* regulated the expression, location (cytoplasmic or transmembrane) and activity of two key components in the distal salt reabsorption machinery, sodium chloride channels sodium-potassium-chloride-cotransporter 2 (NKCC2) and sodium-chloride cotransporter (NCC) [16]. The reasons for the transient phenotype still remain unclear. However, two mechanisms were suggested, hypoxia and the presence in developing kidneys of low phosphodiesterase activity that favours an increase in cyclic adenosine monophosphate (cAMP) concentration [16].

We hypothesized that MAGE proteins may play a role in kidney pathophysiology, given the kidney phenotype of human MAGED2 mutations, and that understanding the regulation of MAGE gene expression may provide insights into their role in kidney injury and as potential therapeutic targets. Given that during kidney injury, both kidney local pO_2 and cAMP levels may change, understanding the regulation and function of MAGED2 during kidney injury may provide new insights into the pathophysiology of AKI. The present study aimed at characterizing the expression and regulation of MAGE genes in normal kidneys and during kidney disease using a systems biology approach.

MATERIALS AND METHODS

Figure 1 summarizes the overall experimental approach. MAGE gene and protein expression were explored by kidney tissue transcriptomics and proteomics and *MAGED2* was selected for further study as being the most differentially regulated gene during experimental kidney injury.

Experimental kidney injury

Studies were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Folic acid nephropathy is a classical model of AKI that shares several features with human AKI, including tubular cell death, compensatory tubular cell proliferation, activation of an inflammatory response and eventual progression to mild fibrosis [19–22]. Indeed, folic acid nephropathy has been reported in humans [23]. C57/BL6 WT female mice (12- to 14-week-old) from the IIS-Fundación Jiménez Díaz animal facilities received a single intraperitoneal injection of folic acid (Sigma, St Louis, MO, USA) 250 mg/kg in 0.3 mol/L sodium bicarbonate or vehicle and were sacrificed 24 or 72 h after injection. This model results in increased serum creatinine, macrophage infiltration and gene expression of mediators of inflammation [including the cytokine tumour necrosis factor-like weak inducer of apoptosis (TWEAK) and markers of tubular cell injury, such as kidney injury molecule-1 (Supplementary data, Figure S1)], as previously extensively described [22, 24–28]. Indeed, targeting the TWEAK/Fn14 system is nephroprotective in this model [22, 24–28]. Kidneys were perfused *in situ* with cold saline before removal. Half-kidney from each mouse

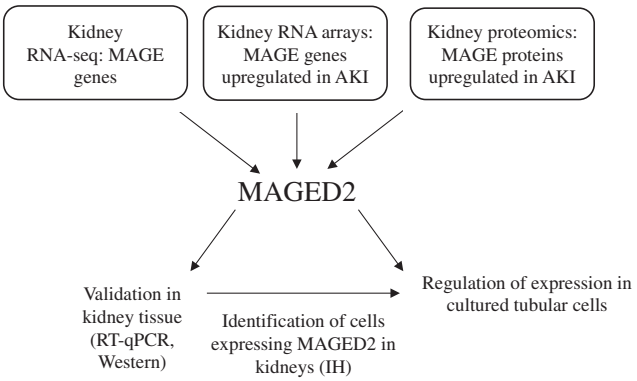

FIGURE 1: Overall experimental approach. IH, immunohistochemistry.

was fixed in buffered formalin, embedded in paraffin and used for immunohistochemistry and the other half was snap-frozen in liquid nitrogen for RNA and protein studies in the model used for transcriptomics arrays and for other studies. A different model ($n = 6$ vehicle and $n = 6$ AKI mice, 24 h of follow-up) was used for proteomics studies. Two sets of folic acid experiments were performed. Three additional healthy adult mice were sacrificed and processed as above for RNA-seq studies.

A different model of AKI was induced by the intraperitoneal injection of a single dose of 25 mg/kg cisplatin (Sigma) dissolved in 0.9% saline. The cisplatin dose was based on literature analysis and results of preliminary experiments, showing renal function impairment at Day 3 after cisplatin injection [29].

Cell cultures

Mouse cortical tubule cells are a cultured line of tubular epithelial cells harvested originally from the renal cortex of Swiss Jim Lambert mice and have been extensively characterized [30]. They were cultured in Roswell Park Memorial Institute 1640 (GIBCO, Grand Island, NY, USA), 10% decomplemented foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, in 5% CO_2 at 37°C and serum-deprived for 24 h before studies. Recombinant human soluble TWEAK (Millipore, Billerica, MA, USA) was added at a final concentration of 100 ng/mL based on prior dose–response studies [31].

RNA arrays

Transcriptomics arrays of kidney tissue were performed at Unidad Genomica Moncloa, (Fundacion Parque Cientifico, Madrid, Spain) using Affymetrix microarrays following the manufacturer's protocol. Image files were initially obtained through Affymetrix GeneChip Command Console Software. Subsequently, Robust Multichip Analysis was performed using the Affymetrix Expression Console Software. Starting from the normalized Robust Multichip Analysis, the Significance Analysis of Microarrays was performed using the limma package (Babelomics, http://www.babelomics.org) and a false discovery rate of (FDR) 5% to identify genes that were significantly differentially regulated between the analysed groups. Three vehicle- and three folic acid-injected AKI mice were studied at the 24 h time-point following injection of folic acid or vehicle. The transcriptomics array result has been previously reported [32–34]. In all, 28 361 transcripts were identified, of which 3485 were differentially regulated (FDR <0.05).

RNA-Seq

Kidneys from three adult mice were used for these studies. From 1 µg total kidney RNA, the PolyA+ fraction was purified and randomly fragmented, reverse transcribed to double-stranded complementary DNA (cDNA) and processed through enzymatic treatments of end-repair, dA-tailing and ligation to adapters as in Illumina's 'TruSeq Stranded mRNA Sample Preparation Part # 15031047 Rev. D' kit. The adapter-ligated library was completed by polymerase chain reaction (PCR) with Illumina PE primers (eight cycles). The resulting purified cDNA library was applied to an Illumina flow cell for cluster generation and sequenced for 50 bases in a single-read format (Illumina HiSeq 2000).

Next-generation sequencing data analysis was performed with the nextpresso pipeline [35]. Sequencing quality was first checked with FastQC [35]. Reads were then aligned to the mouse genome (GRCm38/mm10) with TopHat-2.0.1050, using Bowtie 1.0.051 and Samtools 0.1.1952, allowing two mismatches and five multihits. Transcripts assembly and estimation of their abundance were calculated with Cufflinks 2.2.150, using the mouse genome annotation data set (GRCm38/mm10) [35]. In all, 23 284 transcripts were identified (Supplementary data, Table S1). Transcripts with fragments per kilobase million (FPKM) expression values lower than 0.05 were considered not expressed and were excluded from further analysis.

Molecular interaction analysis

Analysis of potential molecular interactions of MAGED2 was performed using the IntAct database (www.ebi.ac.uk/intact; 20 May 2018, date last accessed) and focused on molecules physically interacting with MAGED2.

Quantitative reverse transcription-PCR (qRT-PCR)

A quantity of 1 µg RNA isolated by Trizol (Invitrogen, Paisley, UK) was reverse transcribed with High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method [29]. Expression levels are given as ratios to glyceraldehyde 3-phosphate dehydrogenase. Predeveloped primer and probe assays were from Applied Biosystems.

Kidney proteomics

Renal cortex from six mice was obtained 24 h after induction of folic acid AKI or vehicle administration. A label-free shotgun proteomic approach was used to identify and quantify modulated proteins. This resulted in 6564 identified proteins, including MAGED2. This technique and global analysis results have been previously described in detail [29, 36] and more extensive information is provided as Supplementary Methods.

Sodium dodecyl sulphate-polyacrylamyde gel electrophoresis and western blot

Sodium dodecyl sulphate-polyacrylamyde gel electrophoresis (SDS-PAGE) was performed using the Mini-PROTEAN (Bio-Rad) kit. In this study, 12% and 10% polyacrylamide gels were used for running and stacking gels, respectively [29]. Blots were blocked with 5% skimmed milk in tris-buffered saline (TBS) containing 0.5% Tween-20 (TBS-T) for 1 h. The primary antibody was rabbit polyclonal anti-MAGED2 (1:1000, Proteintech) in 5% skimmed milk in TBS-T. The blot was incubated overnight at 4°C, washed with TBS-T and incubated with anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, GE Healthcare) in 5% skimmed milk in TBS-T for 1 h at room temperature. After washing in TBS-T, blots were developed with the chemiluminescence method by rinsing the blot in Crescendo Western HRP substrate (Luminata, Merck Milipore Lifescience). Images were obtained using Amersham Imager 600 equipment (GE Healthcare). Blots were then incubated with monoclonal anti-mouse α-tubulin antibody (1:5000, Sigma) overnight at 4°C and, after washing, with anti-mouse IgG antibody peroxidase-conjugated (1:2000 GE Healthcare) for 1 h at room temperature, both in skimmed milk 5% TBS-T.

Immunohistochemistry

Immunohistochemistry was carried out as previously described on paraffin-embedded 3-µm thick kidney tissue sections [29]. Tissue sections were incubated in peroxidase blocking solution (11:10:1 methanol/distilled water/hydrogen peroxide) for 30 min at room temperature and in bovine serum albumin 4%/rabbit serum 10% in phosphate-buffered saline for 1 h at room temperature to prevent non-specific binding. Kidney sections were incubated overnight at 4°C with rabbit polyclonal anti-MAGED2 (1:100, Proteintech), anti-NCC and anti-NKCC2 (1:100, Abcam) or rat polyclonal anti-F4/80 antigen (1:50; Serotec, Oxford, UK) followed by appropriate biotin-labelled secondary antibodies for 1 h at room temperature. Tissue sections were then incubated with ABComplex (Dako kit, Agilent Technologies) and developed with 3,3′-diaminobenzidine (Dako kit, Agilent Technologies). Sections were counterstained with Carazzi's haematoxylin, dehydrated and mounted with DPX glue. Negative controls included incubation with a non-specific IgG of the same isotype as the primary antibody. Staining was evaluated by a quantitative scoring system, using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) in 10 randomly selected fields (×20) per kidney. Samples were examined in a blinded manner.

Human nephrectomy samples were obtained from the IIS-Fundacion Jimenez Diaz Biobank (two males and one female from 61- to 76-year-olds with serum creatinine <1.0 mg/dL, negative dipstick proteinuria and preserved kidney structure in optical microscopy, and a 74-year-old male with serum creatinine 1.6 mg/dL, 50 mg/dL dipstick proteinuria and signs of tubular injury and interstitial fibrosis), in addition to renal biopsy samples from patients with IgA nephropathy and acute tubular necrosis (male, 48-year-old), tubulointerstitial nephritis (female, 70-year-old) and antibody-mediated transplant rejection (male, 46-year-old).

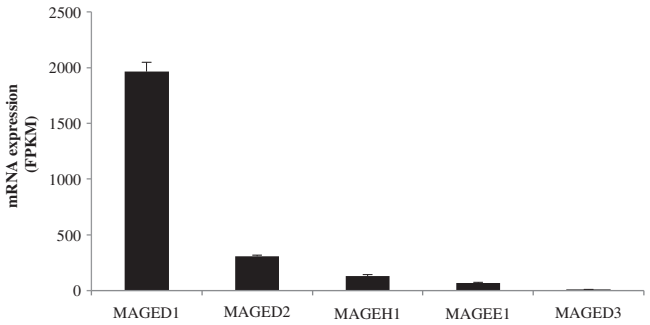

FIGURE 2: Expression of MAGE genes in healthy adult mouse kidney assessed by RNA-Seq. No or very low expression (signal <1.0 FPKM) was found for MAGE genes not shown. Complete data are presented in Supplementary data, Table S1.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 statistical software (IBM, Armonk, NY, USA). Results are expressed as mean ±SEM. Significance at the $P < 0.05$ level was assessed by Student's *t*-test for two groups of data (AKI) and analysis of variance for three of more groups (cell cultures) with Bonferroni correction. For cultured cells, controls were studied at 3 and 24 h and stimulated cells at 3, 6 and 24 h. Short time-points (3–6 h) were compared with the 3-h time-point and the long time-point (24 h) was compared with the 24-h control.

RESULTS

Expression of MAGE genes in mouse healthy adult kidney

The expression of MAGE genes in healthy adult mouse kidney was assessed by RNA-Seq. Five MAGE genes (*MAGED1*, *MAGED2*, *MAGED3*, *MAGEH1*, *MAGEE1*) were expressed at the mRNA level in healthy adult mouse kidneys, as assessed by RNA-Seq (Figure 2 and Supplementary data, Table S2). The highest level of expression was for *MAGED1* followed by *MAGED2*, while *MAGED3* was expressed at very low levels.

Expression of MAGE genes during AKI in mice

The expression of MAGE genes during AKI was approached from both a kidney transcriptomics and a kidney proteomics point of view.

Kidney transcriptomics identified two significantly upregulated (FDR <0.05) MAGE genes 24 h after AKI induction by folic acid administration: *MAGED2* and *MAGEB3* (Figure 3A and Supplementary data, Table S3). However, overall *MAGEB3* expression remained very low since there was barely any constitutive expression. Of the MAGE genes expressed by normal adult kidneys at the mRNA level as assessed by RNA-Seq, kidney proteomics only identified *MAGED2* as present in at least one of six (17%) vehicle-injected murine kidney samples. By contrast, it was detected in five of the six (83%) AKI samples 24 h after folic acid administration (Supplementary data, Table S4).

MAGED2 expression in experimental AKI

In the initial systems biology approaches involving kidney transcriptomics and proteomics, *MAGED2* was one of two MAGE mRNAs significantly upregulated in AKI and the only kidney-expressed (meaning the mRNA was constitutively expressed) MAGE protein observed upregulated by proteomics. Thus, the next step was to validate the systems biology data by qRT-PCR, western blot and immunohistochemistry.

The qRT-PCR technique confirmed the upregulation of MAGED2 mRNA in kidneys from mice with folic acid-induced AKI (Figure 3B). The increased kidney expression of MAGED2 protein observed by proteomics was also confirmed by western blot (Figure 3C). Additionally, *MAGED2* mRNA (Figure 3D) and protein (Figure 3E) were also increased in kidneys from mice with AKI induced by cisplatin. The increased kidney expression of MAGED2 protein observed by proteomics and western blot in murine AKI was localized to tubular cells by immunohistochemistry in mice injected with cisplatin (Figure 4A and Supplementary data, Figure S2A) or folic acid (Supplementary data, Figure S2B). In human kidneys, basal expression of MAGED2 protein was observed in kidney tubular cells and increased expression was noted in damaged human kidney tubules (Figure 4B and Supplementary data, Figure S3).

TWEAK promotes MAGED2 expression in cultured tubular cells

Since during AKI, kidney MAGED2 expression is increased and localized to tubular cells, we assessed MAGED2 expression in cultured tubular cells and explored potential regulators of this expression. TWEAK is an inflammatory cytokine known to promote kidney injury and tubular cell stress [24, 37, 38].

Adding TWEAK to the culture medium caused a transient (3 and 6 h of incubation) increase of *MAGED2* gene expression at the mRNA level (Figure 5A). TWEAK also increased MAGED2 protein levels with a temporal pattern similar to the mRNA levels (Figure 5B). The transient increase in protein levels suggests that the MAGED2 protein half-life is short.

Serum deprivation promotes MAGED2 expression in cultured tubular cells

Serum deprivation leads to cell quiescence by deprivation of mitogenic factors. However, it also deprives survival factors and this may cause cell stress and even death [39, 40]. Cultured tubular cells incubated in serum-free medium upregulated *MAGED2* gene expression at the mRNA level within 3 h and the increased expression persisted for at least 24 h (Figure 5C), In addition, serum deprivation increased MAGED2 protein levels from 3 h on, following a pattern similar to mRNA expression (Figure 5D and Supplementary data, Figure S4).

Potential functions of MAGED2 in kidney injury

The MAGED2 regulation of the function of tubular cell sodium chloride channels NCC and NKCC2 has been clearly established [16]. In this regard, immunohistochemistry suggested upregulation of NCC and NKCC2 immunostaining in kidney tubules during folic acid-induced AKI (Supplementary data, Figure S5), which would be consistent with the recently described function of MAGED2 to protect them from degradation [16]. However, MAGED2 may have additional functions. A molecular interactions map identified a number of potential MAGED2 partners (Figure 6). Of these, 22 were expressed at the mRNA level in the adult mouse kidney and 5 were upregulated during AKI, while 1 was downregulated (Supplementary data, Table S5).

DISCUSSION

The main findings of this study are that several MAGE genes are expressed constitutively in the adult kidney and MAGED2 is upregulated during kidney injury and, specifically, in experimental AKI and human kidney disease. These findings were confirmed by qRT-PCR and western blot, and immunohistochemistry localized MAGED2 expression to kidney tubular cells. Cultured tubular cells increased MAGED2 expression in response to stressors such as serum deprivation or an inflammatory cytokine. In conjunction with recently published data on the role of MAGED2 mutations in hereditary kidney disease, the present data suggest an important role for MAGED2 in kidney injury.

MAGED2 is an ubiquitous protein that is required for normal tubular function during foetal life [6, 16]. Thus, loss of function mutations in MAGED2 are linked to transient antenatal Bartter syndrome [16], indicating a key role of the protein in regulating salt balance, at least in the foetus. We confirmed a prior report of constitutive kidney MAGED2 expression in tubular cells [16], and additionally identified upregulation during kidney injury both in mice and humans. The immunohistochemistry technique used was not sufficiently sensitive to detect MAGED2 protein in normal mouse kidney, although it did detect the protein in tubules during experimental AKI. Furthermore, human studies confirmed constitutive MAGED2 expression in normal human tubules, mainly in distal tubules, but also in proximal tubules, as well as increased tubular MAGED2 during kidney injury. The combination of *in vivo* and cell culture experiments suggest that increased gene expression may be contributing to the higher levels of MAGED2 protein during kidney injury.

The constitutive function of MAGED2 in the adult kidney has not been characterized since MAGED2 deficiency caused only transient prenatal disease in humans. Prenatally, MAGED2 appears to have as a key function the regulation of NCC and NKCC2 expression and correct location, likely by protection against degradation in the endoplasmic reticulum and by enhancing NCC activity and NKCC2 membrane translocation to the apical membrane [16]. During AKI or CKD, local inflammation and cell injury and death are associated to impaired kidney function and adverse consequences such as volume overload and hypertension [41, 42]. Given the already characterized role of *MAGED2* in the regulation of sodium transporters, a role of MAGED2 overexpression in facilitating sodium reabsorption and contributing to volume overload and hypertension by facilitating the activity of NCC and NKCC2 may be hypothesized. However, this hypothesis should be addressed by functional *in vivo* studies. In tubular cells, MAGED2 may bind to the molecular chaperone Hsp40 and to

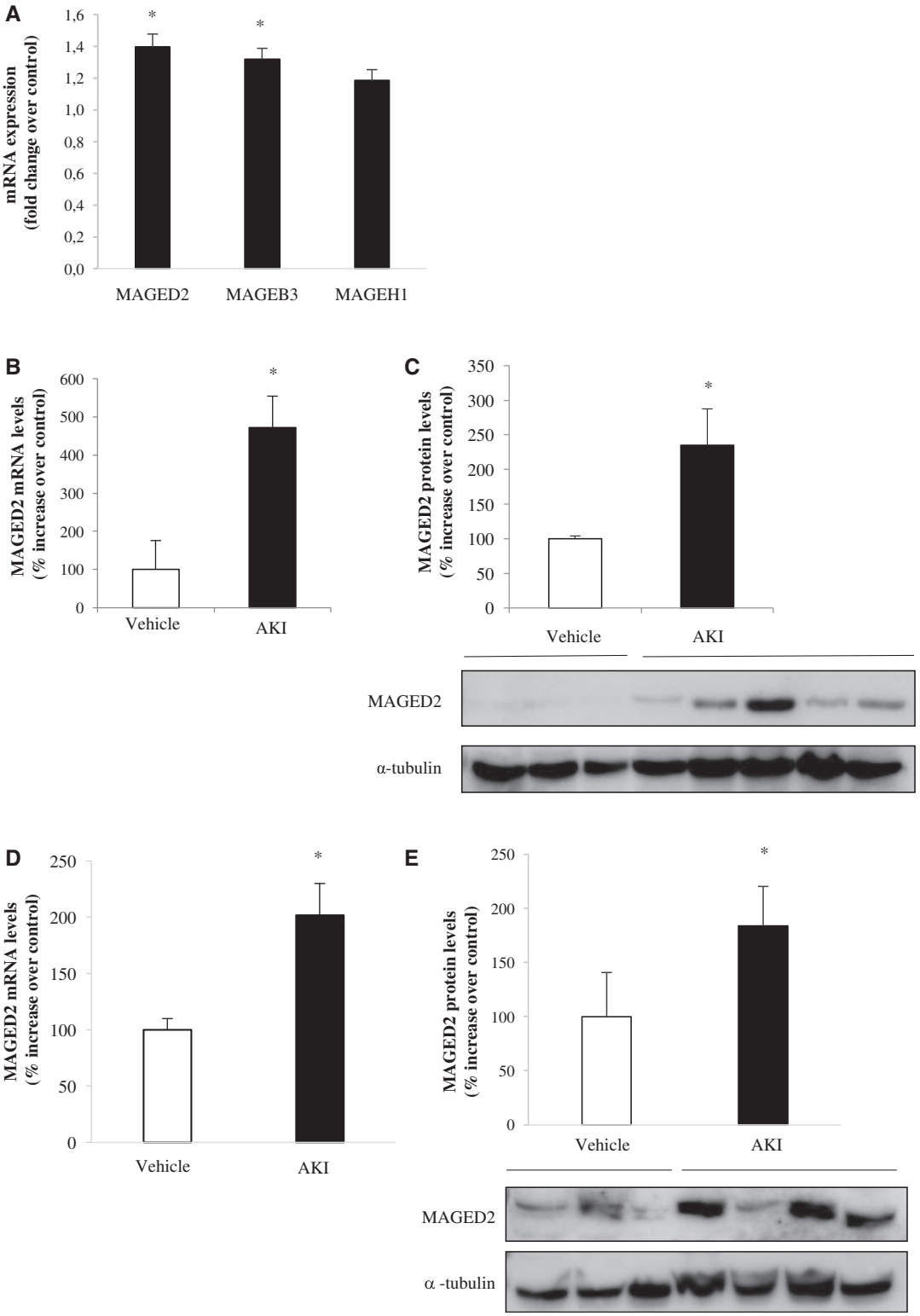

FIGURE 3: MAGE gene expression during AKI. (**A**) Kidney MAGE mRNA levels as assessed by RNA arrays. Only differentially expressed MAGE genes with a P-value <0.05 are shown. Asterisk denotes an FDR <0.05. Data expressed as mean±SEM fold-change of three AKI mice versus three control vehicle mice, 24 h after injection of folic acid or vehicle. Complete data are presented in Supplementary data, Table S3. (**B**). qRT-PCR assessment of kidney mRNA levels. Mean±SEM of three vehicle control and five AKI mice, 72 h after injection of folic acid or vehicle. *P < 0.05. (**C**) Western blot of protein extracted from murine kidneys. Representative image and quantification. Mean±SEM of three vehicle control and five AKI mice, 72 h after injection of folic acid or vehicle. *P < 0.05. (**D**) qRT-PCR assessment of kidney mRNA. Mean±SEM of four vehicle control and six AKI mice, 72 h after injection of cisplatin or vehicle. *P < 0.05. (**E**) Western blot of protein extracted from murine kidneys. Representative image and quantification. Mean±SEM of three vehicle control and four AKI mice, 72 h after injection of cisplatin or vehicle. *P < 0.05.

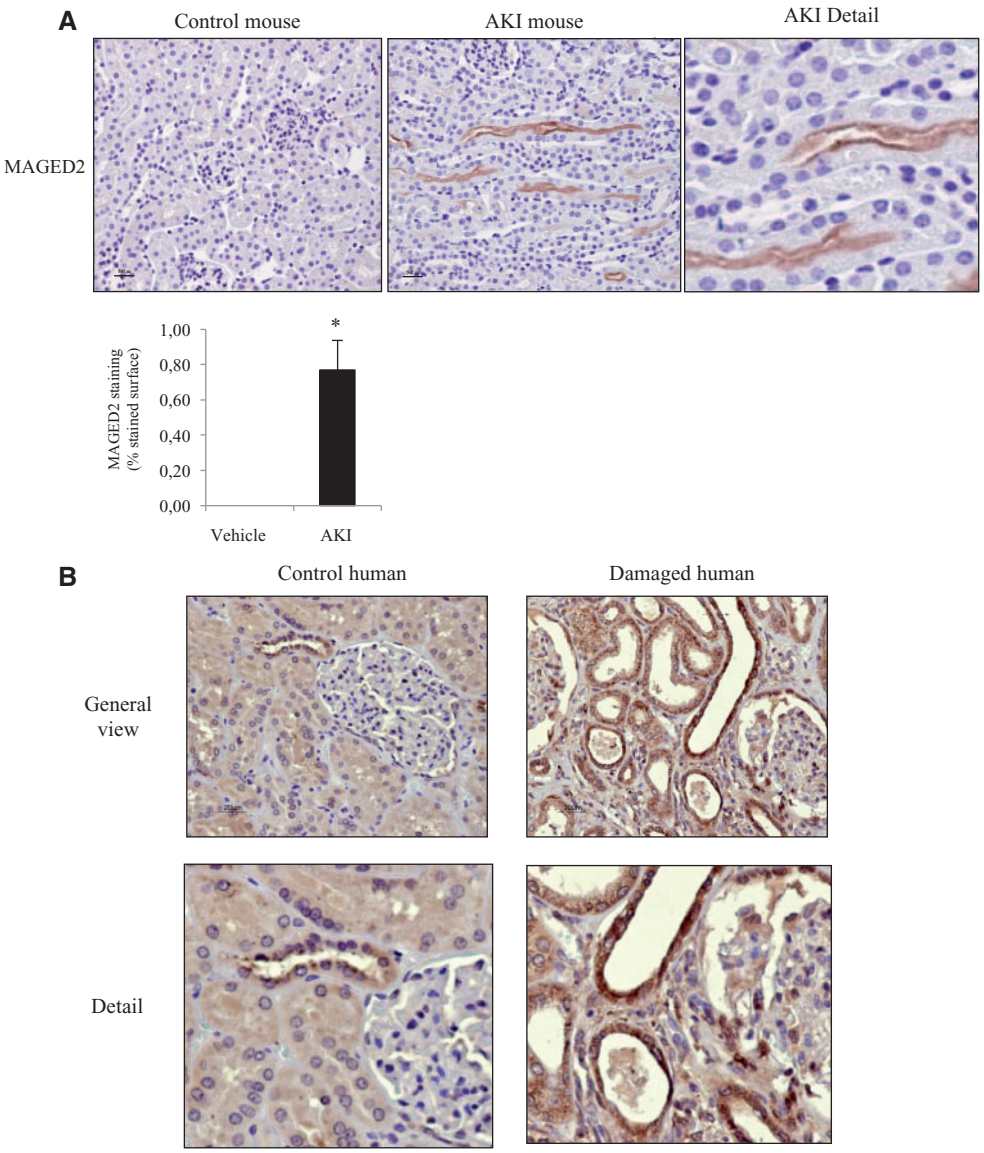

FIGURE 4: Kidney immunohistochemistry localized MAGED2 to tubular cells in murine AKI and damaged human kidney. (**A**) Murine vehicle control and AKI kidneys, sampled 72 h following administration of vehicle or cisplatin. MAGED2 protein was not detected in healthy murine kidneys by the technique used but was present in tubules during AKI. Representative images and quantification. Original magnification ×20. (**B**) Human control and damaged kidneys, MAGED2 protein is expressed in tubules in control human kidneys and tubular expression is increased in damaged human kidney. Original magnification ×40.

cytoplasmic Gs-alpha [43]. Enhancing the chaperone activity of Hsp40 may protect certain proteins, including but not limited to sodium transporters, from endoplasmic reticulum-associated degradation. Regarding NKCC2 and NCC, protection from degradation appears to be especially important during foetal life, but we may speculate that the injured kidney milieu may sensitize these or other proteins to endoplasmic reticulum-associated degradation, increasing the relevance and/or need for MAGED2 activity. In this regard, tubular immunostaining for NKCC2 and NCC was more apparent during experimental AKI. Binding to Gs-alpha may contribute to activate adenylate cyclase and increase cAMP levels. In their *NEJM* manuscript, Laghmani *et al.* speculated that lower cAMP or oxygen levels in foetal kidneys may increase the need for MAGED2 [16]. In this regard, during kidney injury these conditions may also exist. Low urinary cAMP excretion during AKI was described >40 years ago and shown to be related to decreased adenylate cyclase activity [44, 45]. Low oxygen levels are thought to be key contributors to both AKI of diverse aetiologies and to CKD progression, in part due to capillary rarefaction [46]. Thus, it is likely that during kidney injury, the pathophysiological impact of MAGED2 may be increased as it is during foetal development. The potential functions of MAGED2 in kidney cells may go beyond the already characterized regulation of electrolyte transporters. Thus, MAGED2 interacts with proteins involved in the stress response, signal transduction, cell and molecular turnover and gene transcription regulation, some of which were differentially expressed during AKI.

In cultured cells, MAGED2 expression was upregulated by stressors that may cause cell death (e.g. serum deprivation) [19]

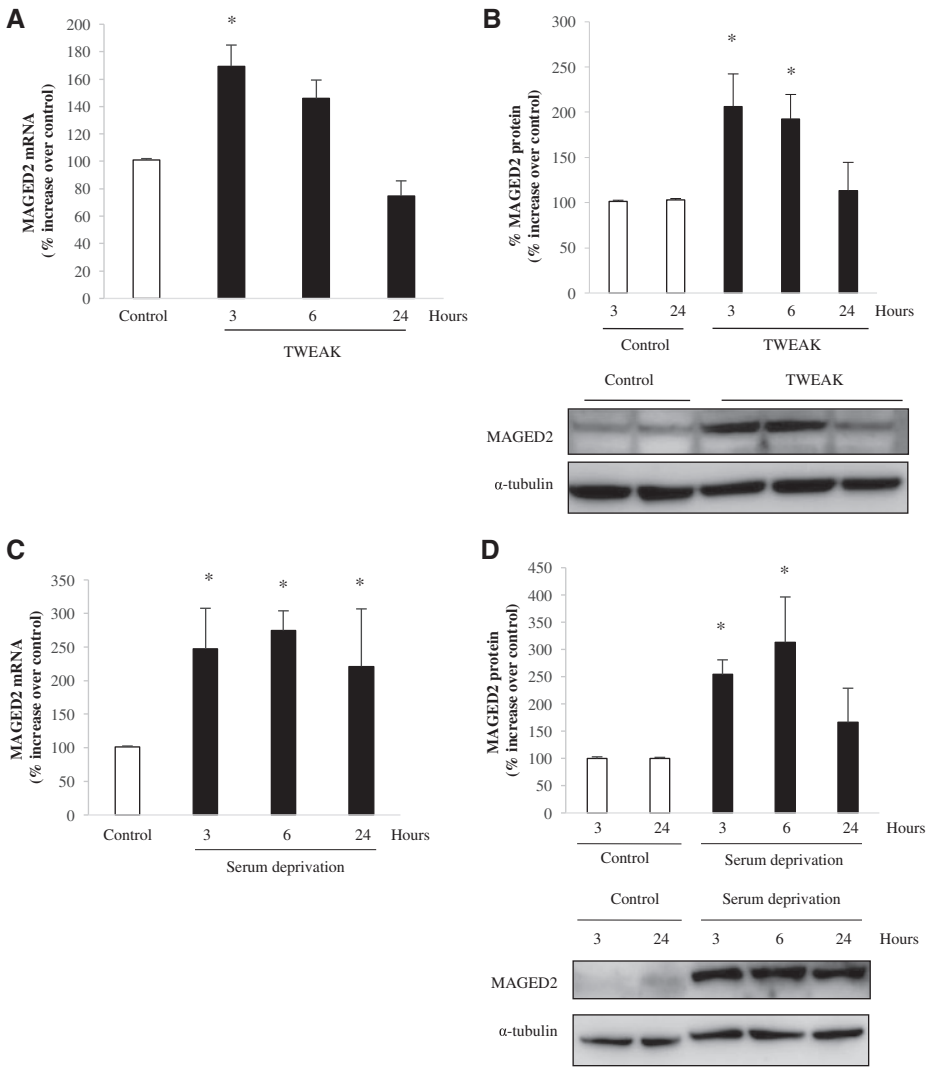

FIGURE 5: TWEAK and serum deprivation increase MAGED2 mRNA and protein expression in cultured tubular cells. qRT-PCR and western blot. (**A**) TWEAK stimulation significantly increased MAGED2 mRNA levels over control values at 3 h. Mean±SEM of three independent experiments. *$P < 0.005$ versus control. (**B**) TWEAK stimulation significantly increased MAGED2 protein levels. Mean±SEM of three independent experiments. *$P < 0.05$ versus control. (**C**) Serum deprivation significantly increased MAGED2 mRNA levels over control values from 3 to 24 h. Mean±SEM of three independent experiments. *$P < 0.05$ versus control. (**D**) Serum deprivation increases MAGED2 protein expression in cultured tubular cells as assessed by western blot of total protein extracts. Mean±SEM of three independent experiments. *$P < 0.05$ versus control.

or a proinflammatory response and predisposition to cell death in an inflammatory environment (TWEAK) [24, 37]. In both cases, both MAGED2 mRNA and protein were upregulated, pointing to a potentially shared mechanism of upregulation. Whether MAGED2 may contribute to withstand stress or is part of a deleterious response or has additional functions should be clarified by functional studies. In this regard, there are examples of proteins upregulated during stress that contribute to cell injury and death (e.g. the nuclear factor-κB-inducing kinase NIK/MAP3K14) [29] or protect from cell injury or death (e.g. Bcl3 or Hsp27/HSPB1) [37, 47, 48].

The present work has several strengths. A multi-omics approach was used to find MAGE genes potentially involved in kidney injury. Thus, both transcriptomics and proteomics data were consistent in identifying MAGED2 as overexpressed during AKI. Moreover, the data were validated at the mRNA and protein expression levels, in culture and *in vivo*, in different models of murine AKI and in humans. The diverse techniques and experimental settings all pointed to increased MAGED2 expression during kidney injury.

Some limitations should be acknowledged. Label-free mass spectrometry analysis of proteinaceous samples can at times result in ambiguous values, depending also on statistical and mathematical procedures and threshold cut-off levels. Yet, corroboration and validation using unrelated and methodologically different techniques should be performed in combination with any mass spectrometric detection methodology, such as the ones used in this study, which in turn substantiates not only the specific mass spectrometry findings, but also enables exploration of related molecules that have not been detected. This detection limitation by mass spectrometry is highly dependent on the composition of the measured sample as well as on the type

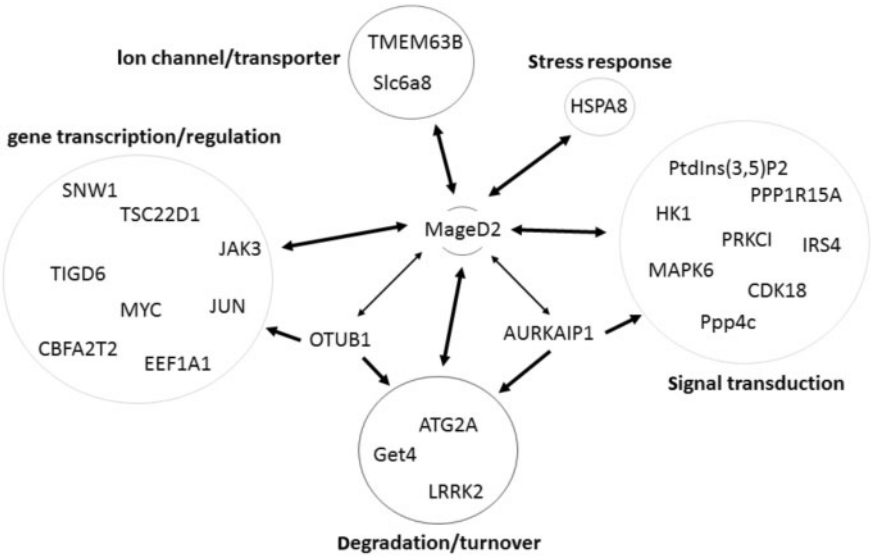

FIGURE 6: Schematic representation of MAGED2 interactions. Information regarding molecules physically interacting with MAGED2 were retrieved from the IntAct database (www.ebi.ac.uk/intact) and grouped according to apparent biological involvements.

of machine and technique used. Also, the function of MAGED2 was not specifically explored. Functional studies should be designed in which MAGED2 is overexpressed or genetically targeted in cultured tubular cells and *in vivo* in order to explore its function during kidney injury.

In conclusion, MAGED2 mutations have been recently described to cause kidney disease. Taken together with our data identifying MAGED2 as upregulated at the mRNA and protein level in AKI as well as evidence that this increased expression is localized to tubular cells *in vivo* and that tubular cell stressors increase the expression of MAGED2 in cultured tubular cells, this information points to an important role of MAGED2 in kidney disease that should be dissected further by carefully designed functional approaches in cell culture and *in vivo*.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

M.D.S.-N. and A.O. designed the study and directed the research. M.D.S.-N. and L.V.-R. performed animal experiments. L.V.-R., L.C. and M.A. performed cell culture studies. H.H. and H.M. performed bioinformatics analysis. M.D.S.-N., L.V.-R. and L.C. collaborated in the analysis of the results. P.C.-O. performed human studies. All authors critically reviewed for content and approved the final version.

CONFLICT OF INTEREST STATEMENT

None declared.

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