Although the classic view of diabetic nephropathy (DN) has focused on events leading to glomerular dysfunction, the gradual decline of renal function in later stages of DN is invariably associated with tubulointerstitial fibrosis and tubular atrophy (1). Indeed, tubulointerstitial fibrosis and tubular atrophy appear to be better predictors of late-stage renal disease progression than glomerular pathology (2–5). For example, examination of nephrons from proteinuric diabetic patients shows that 71% of glomeruli display glomerulotubular junction abnormalities and 8–17% of glomeruli are atubular glomeruli (6,7).

The mechanisms underlying tubular atrophy are incompletely delineated. Studies have shown that high glucose (HG) concentrations are associated with increased reactive oxygen species (ROS) production, which inhibits proximal tubular function and induces apoptosis (8–10). Apoptosis has been detected in renal proximal tubular cells (RPTCs) of diabetic mice (11,12) and rats (13,14) as well as in RPTCs of diabetic patients (15–17), suggesting that tubular apoptosis may precede tubular atrophy in atubular glomeruli. Although the link between ROS and tubular apoptosis seems clear, little is known about the genes involved in HG-induced RPTC apoptosis or ROS generation.

We previously reported that HG enhances angiotensinogen (Agt) gene expression via ROS generation in rat RPTCs in vitro (18,19) and that in vivo overexpression of rat Agt in RPTCs induces hypertension, albuminuria, and RPTC apoptosis in diabetes (20). Conversely, we also reported that RPTC-selective overexpression of catalase (CAT) attenuates ROS generation, tubulointerstitial fibrosis, and tubular apoptosis as well as proapoptotic gene expression in diabetic mouse kidneys in vivo (21,22). These data suggest that ROS generation may be directly or indirectly responsible for RPTC apoptosis in diabetes.

We now report that Bcl-2–modifying factor (Bmf), a proapoptotic gene that we identified via DNA chip microarray analysis, is differentially upregulated in RPTCs of db/db mice; we also validated this observation by immunohistochemistry and real-time quantitative PCR (qPCR). We further show enhanced Bmf expression in the RPTCs of mice with streptozotocin (STZ)-induced diabetes as well as in the kidneys of patients with diabetes. Finally, we found that Bmf overexpression enhances RPTC apoptosis and that HG in vitro induces Bmf mRNA expression via ROS generation and transforming growth factor-β1 (TGF-β1) expression.
Regulated protein (KAP) promoter responsive to testosterone stimulation was

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Real-time qPCR assays for gene expression.

Louisville, KY). The plasmid pKAP2 containing the kidney-speci-

Real-time qPCR was performed according to previously described

(i.e., db/db vs. db/+Tg mice). A list of nor-

DNA microarray analysis.

Mouse RPT isolation and DNA microarray analysis. Animals were killed at

mouse RPTs were immediately processed for
total RNA isolation and gene chip microarray analysis. Briefly, total RNAs from
three mice from each group were purified and reverse-transcribed into cDNA, which, in turn, served as the template for the generation of biotin-labeled cRNA (Enzo kit, Affymetrix, Inc., Santa Clara, CA), and then hybridized to Affymetrix Mouse Genome 430 2.0 microarray chips (Affymetrix, Inc.), according to the manufacturer’s protocol. Affymetrix Mouse 430 A 2.0 chips contain ~45,000 probe sets, corresponding to more than 39,000 mouse transcripts. The data were analyzed using GeneChip Operating Software (Affymetrix) and the LIMMA package (version 2.10.5). The Bioconductor Library (release 2.0) were used for data analysis (27–30). The GCRMA (Gene Chip Robust Multi-array Average) algorithm was used for back-
ground correction of the data (31), and a linear model fit was undertaken on different contrasts representing the desired group comparisons (i.e., db/db vs. homozygous db/db+ mice, db/db+ mice vs. db/db CAT-Tg mice). A list of nor-
malized data (genes linked to the apoptotic pathway) from all probe sets rep-

Classifying differentially regulated genes qPCR

Statistical analysis.

RESULTS

Microarray analysis. Normalized data of the different

The Gene Ontology database (32) for the different probe sets

\textit{Transfections, apoptosis, and caspase-3 activity assays.} The pCMV-Myc mammalian expression vector containing N-terminally Myc-tagged rat Bmf (Supplementary Fig. 1) was transiently transfected into RPTCs using Lip


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higher in RPTCs of db/db mice compared with db/m+ and db/db CAT-Tg mice.

**Validation of Bmf mRNA expression in mouse RPTs by real-time PCR.** To validate the results obtained by DNA microarray, real-time qPCR was performed using primers specific for mouse Bmf mRNA. Figure 1A displays the results of Bmf mRNA expression in freshly isolated RPTs from db/m+, db/db, and db/db CAT-Tg mice. The baseline expression of Bmf mRNA in db/db mice was sixfold higher than in db/m+ mice (P < 0.005). This increase was significantly attenuated in db/db CAT-Tg mice (P < 0.05).

**Validation of Bmf expression by immunohistochemistry.** We previously reported TUNEL staining on kidney sections of db/m+, db/m+ CAT-Tg, db/db, and db/db CAT-Tg mice and showed that apoptotic cells were found in RPTCs of db/db mice but not in db/m+, db/m+ CAT, and db/db CAT-Tgs (22). To validate whether Bmf expression was increased in RPTCs of 20-week-old T2DM db/db mice, immunohistochemical analysis was performed using an anti-Bmf antibody. Increased immunostaining for Bmf was observed in the RPTCs of diabetic db/db mice (Fig. 1B, b) compared with the RPTCs of non-diabetic db/m+ control mice (Fig. 1B, a). CAT overexpression effectively attenuated Bmf expression, as observed in RPTCs of db/db CAT-Tg mice (Fig. 1B, c). No immunostaining was observed with nonimmune control serum in db/db mice (Fig. 1B, d). Quantification of Bmf immunostaining confirmed enhanced Bmf expression in RPTCs of db/db mice (Fig. 1C). Similarly, kidneys from adult mice with STZ-induced diabetes also exhibited enhanced Bmf immunostaining in RPTCs (Fig. 2A, b and B) compared with nondiabetic littersmates (Fig. 2A, a and B). Treatment of diabetic mice with insulin reduced Bmf expression to control levels (Fig. 2A, c and B). No immunostaining was observed in sections treated with nonimmune control serum (Fig. 2A, d). These results were further validated by performing real-time qPCR for Bmf using RNA isolated from RPTCs of STZ-induced diabetic mice. Figure 2C shows a significant increase (P < 0.05) in Bmf mRNA expression levels in STZ-induced diabetic mice compared with control and insulin-treated STZ-injected mice.

**HG induces ROS generation and Bmf mRNA expression in RPTCs in vitro.** Immortalized rat RPTCs were cultured in normal glucose or HG medium with or without rotenone, CAT, DPI, or apocynin. Cells were then harvested to assess ROS generation with the lucigenin assay, and RNA was isolated for real-time qPCR analysis. RPTCs cultured in HG produced significantly higher amounts of ROS than RPTCs cultured in normal glucose (Fig. 3A), and these increases could be markedly attenuated or inhibited by rotenone, CAT, DPI, or apocynin. Furthermore, RPTCs cultured in HG medium exhibited fourfold higher Bmf mRNA expression than RPTCs cultured in normal glucose medium (P < 0.01; Fig. 3B). The HG-stimulated increases in Bmf mRNA expression were inhibited by rotenone, CAT, DPI, and apocynin.

**TGF-β1 upregulates Bmf mRNA expression in rat RPTCs.** To investigate the mechanism(s) of HG-stimulation of Bmf expression, RPTCs were cultured with active human TGF-β1 in normal glucose medium. TGF-β1 increased Bmf mRNA expression in a concentration-dependent manner (Fig. 3C). Conversely, knockdown of TGF-β1 with siRNA attenuated HG-stimulation of Bmf mRNA expression in RPTCs (Fig. 3D).

**Cloning and amino acid sequence of rat Bmf.** We cloned Bmf cDNA from rat RPTCs (Wistar strain) by conventional RT-PCR. Rat and mouse Bmf consists of 185 amino acids and is 98.9% homologous. Rat Bmf cDNA was then subcloned into the pCMV-Myc mammalian expression vector, which fused an NH2-terminal e-Myc epitope tag (Supplementary Fig. 1A and B).

**Bmf overexpression leads to activation of caspase-3 and RPTC apoptosis.** To study the role of Bmf in apoptosis, rat RPTCs were transiently transfected with NH2-terminally Myc-tagged Bmf or an empty vector, and

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**Table 1**

Proapoptotic genes upregulated in microarray chips of db/db vs. db/m+ and db/db vs. db/db CAT-Tg mice overexpressing CAT*

| Probe set ID   | Gene title                          | db/db vs. db/m+ | db/db vs. db/db CAT-Tg |
|---------------|-------------------------------------|-----------------|------------------------|
| 1450313_at    | Baculoviral IAP repeat-containing 4 | 1.28 0.0039     | 1.29 0.0038            |
| 1454880_at    | Bcl2-modifying factor               | 3.07 0.0099     | 3.07 0.0098            |
| 1449297_at    | Caspase 12                          | 1.82 0.0069     | 1.81 0.0070            |
| 1431875_at    | E2F transcription factor 1          | 1.19 0.0065     | 1.19 0.0064            |
| 1423602_at    | TNF receptor-associated factor 1    | 1.99 0.0073     | 1.97 0.0074            |

*The GO annotation package was used to select genes involved in the apoptosis process. The probe sets were selected with P < 0.01.

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**Table 2**

Proapoptotic genes downregulated in microarray chips of db/db vs. db/m+ and db/db vs. db/db CAT-Tg mice overexpressing CAT*

| Probe set ID   | Gene title                          | db/db vs. db/m+ | db/db vs. db/db CAT-Tg |
|---------------|-------------------------------------|-----------------|------------------------|
| 1417962_at    | Growth hormone receptor             | −2.92 0.0031    | −3.44 0.0024           |
| 1419592_at    | Unc-5 homolog C (C. elegans)        | −1.53 0.0058    | −1.39 0.0090           |
| 1435369_at    | U box domain-containing 5           | −2.10 0.0012    | −1.84 0.0016           |
| 1451814_at    | Peptidyl-tRNA hydrolase 2           | −1.88 0.0018    | −1.32 0.0087           |
| 1452172_at    | FAST kinase domains 2               | −2.10 0.0013    | −1.60 0.0027           |
| 1460071_at    | Glutathione peroxidase 1            | −1.41 0.0091    | −1.51 0.0063           |

*The GO annotation package was used to select genes involved in the apoptosis process. The probe sets were selected with P < 0.01.
caspase-3 activity was determined in cell lysates. Expression of the fusion protein was confirmed by RT-PCR (Fig. 4A) and anti-Myc immunoblotting (Fig. 4B). Caspase-3 activity was significantly increased in lysates from cells transiently transfected with the Bmf fusion protein compared with lysates from cells transiently transfected with empty vector \((P < 0.005)\). Caspase-3 activity was further augmented

### FIG. 1. Bmf expression is elevated in RPTs of db/db diabetic mice.

A: Real-time qPCR for Bmf mRNA levels in freshly isolated RPTs from db/m, db/db, and db/db CAT-Tg mice. Values were corrected to β-actin. B: Bmf immunohistochemical (IHC) staining in kidney sections (original magnification ×600) from (a) db/m, (b) db/db, and (c) db/db CAT-Tg mice; (d) presents a nonimmune rabbit serum control. G, Glomerulus. C: Quantification of Bmf IHC staining. Values are the mean ± SEM, \(n = 5\)–7 for each group. \(* P < 0.05; ** P < 0.005. N.S., not significant. (A high-quality color representation of this figure is available in the online issue.)

### FIG. 2. Bmf expression is upregulated in RPTs from STZ-induced diabetic mouse kidneys.

A: Bmf immunohistochemical (IHC) staining in kidney sections (original magnification ×600) from nondiabetic control (a), STZ-induced diabetic (b), and insulin-treated STZ-injected mice (c); nonimmune rabbit serum control is also presented (d). B: Quantification of Bmf-IHC. Values are the mean ± SEM, \(n = 4\)–12. C: Real-time qPCR for Bmf mRNA levels in freshly isolated RPTs from control and STZ-induced diabetic and insulin-treated STZ-injected mice. Values were corrected to β-actin. \(* P < 0.05; ** P < 0.01. N.S., not significant. (A high-quality color representation of this figure is available in the online issue.)
when RPTCs were cultured in HG medium ($P < 0.005$; Fig. 4C). Knockdown of Bmf with siRNA reduced HG-induced apoptosis (TUNEL assay) in RPTCs compared with scrambled siRNA (Fig. 4D).

RPTCs transiently transfected with NH2-terminally Myc-tagged Bmf exhibited fourfold increase in TUNEL-positive cells compared with empty vector-transfected cells ($P < 0.005$; Fig. 5A and B). Parallel assays with lysates from transfected cells confirmed the expression of Myc-tagged Bmf (Fig. 5C).

**Bmf interacts with Bcl-2 in rat IRPTCs.** To investigate Bmf interaction with Bcl-2, coimmunoprecipitation experiments of Myc-Bmf with Bcl-2 were performed. Significant increases in coimmunoprecipitated Bcl-2 (Fig. 5D, a) but not Myc-Bmf (Fig. 5D, b) were observed in rat IRPTCs overexpressing Myc-Bmf cultured in HG medium.

**Bmf expression in diabetic human kidneys.** The clinical characteristics of the patients are shown in Supplementary Table VI. All had kidney cancer, which was the reason for the nephrectomies; some had T2DM, but others did not. Immunohistochemistry revealed Bmf expression in the renal distal tubules but not in the renal proximal tubules (RPTs) of the normal portions of human nephrectomy specimens from nondiabetic patients with kidney cancer (Fig. 6A, a–c). However, increased immunostaining for Bmf was observed in RPTs of the normal portions of nephrectomy specimens from patients with kidney cancer who also had diabetes (Fig. 6A, d–f). Interestingly, double immunostaining revealed frequent colocalization of Bmf overexpression in TUNEL-positive apoptotic RPTCs in diabetic kidney but not in nondiabetic kidney (Fig. 6B).

DNA gene chip microarray analysis (Affymetrix Gene Chip HGU 133plus 2 chip) of microdissected nephrons from seven patients with T2DM revealed modestly but significantly enhanced Bmf expression in glomeruli (1.48-fold increase) and tubulointerstitium (1.39-fold increase) compared with 18 control biopsy specimens from kidneys from living donors taken at the time of transplant ($q < 0.01$, where $q$ value is a multiple-testing corrected
FIG. 4. Overexpression of rat Bmf induces caspase-3 activity in rat RPTCs in vitro. 

A: RPTCs were transiently transfected by lipofectamine 2000 with the empty vector, pCMV-Myc or plasmid containing NH2-terminally tagged rat Bmf cDNA (pCMV-Myc rat Bmf). Expression of the Myc-rat Bmf mRNA was confirmed by conventional RT-PCR and quantified by densitometry.

B: Anti-Myc immunoblotting was also performed. The nonspecific band serves as loading control.

C: Caspase 3 activity in RPTCs transfected with empty vector (pCMV-Myc) or pCMV-Myc rat Bmf in normal glucose (□) and HG (■) media. Values are corrected to lysate protein levels. Values are the mean ± SEM, n = 4–8. ***P < 0.005. UV, ultraviolet.

D: TUNEL images (a–d) and quantification of apoptotic cells (E) in vitro. After transfection with scrambled siRNA (a and c) or Bmf siRNA (b and d) in RPTCs cultured in normal glucose (NG, a and b) or HG (c and d) medium, cells were fixed and subjected to TUNEL (green) and DAPI (blue) staining (original magnification ×200). White arrows indicate TUNEL-positive cells; (e) quantification of TUNEL-positive cells (n = 3 experiments). **P < 0.01; N.S., not significant. (A high-quality color representation of this figure is available in the online issue.)

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FIG. 5. Overexpression of rat Bmf increases TUNEL-positive cells and coimmunoprecipitates (Co-IP) with Bcl-2 in rat RPTCs in vitro. A: RPTCs were transiently transfected with the empty vector, pCMV-Myc, or pCMV-Myc rat Bmf. Cells were incubated for 24 h in 25 mmol/L d-glucose, then fixed and subjected to TUNEL and DAPI staining. TUNEL (green) and DAPI (blue) staining (original magnification ×200) are shown for RPTCs transfected with empty vector and pCMV-Myc rat Bmf. DNase-treated cells serve as TUNEL-positive controls. Cells left untreated with terminal transferase serve as a TUNEL-negative control. B: Quantification of TUNEL-positive cells per field is shown. Values are presented as percentages of TUNEL-positive cells/total cells per field ± SEM (n = 8 or 9). ***P < 0.005. C: Expression of the Myc-rat Bmf fusion protein was confirmed by anti-Myc immunoblotting, with β-actin as the loading control. D: Interaction of Myc-Bmf with Bcl-2 in rat RPTCs: (a) Immunoblotting for Bcl-2 in cytosolic fractions of rat RPTCs before (total lysate, TL) and after Co-IP with anti-Myc; (b) immunoblotting for Myc in cytosolic fractions of rat RPTCs on the same membrane after immunoblotting for anti-Bcl-2 in panel a. The relative density of Bcl-2 or Myc in RPTCs cultured normal glucose (5 mmol/L d-glucose plus 20 mmol/L d-mannitol DMEM) was expressed as controls (100 arbitrary units). Rabbit purified IgG (3 μg) was used as the control for Co-IP experiments. Values are the mean ± SEM for 4 independent experiments. ***P < 0.005; N.S., not significant. □, normal glucose; ■, high glucose medium. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 6. Enhanced Bmf expression in RPTs from human kidneys from patients with diabetes. **A**: Bmf immunohistochemical staining in human kidney sections (original magnification ×200) from three nondiabetic cancer patients (a, patient with papillary variant carcinoma; b, patient with clear cell carcinoma; and c, patient with thyroid-like renal carcinoma) and three diabetic cancer patients (d, patient with papillary variant carcinoma and evidence of DN [nodular with tubular atrophy and interstitial fibrosis]; e, patient with clear cell carcinoma and no evidence of DN; and f, patient with clear cell carcinoma and nephroangiosclerosis with evidence of DN [secondary focal glomerulosclerosis, tubular atrophy, and interstitial fibrosis]). DT, distal tubule; G, glomerulus. **B**: Colocalization of Bmf expression and TUNEL-positive cells in human kidneys. Nondiabetic human kidney (patient I.D. #C,) and diabetic human kidney with DN (patient I.D. #D) were sectioned, subjected to TUNEL assay to visualize apoptotic cells (green), and then incubated with anti-Bmf antibody, followed by anti-goat AlexaFluor 594 to demonstrate Bmf expression (red). Cells staining positively for TUNEL and Bmf appear yellow (merged image). Original magnification ×200. Arrows indicate cells that stained positively for TUNEL and Bmf. G, glomerulus; DT, distal tubule. (A high-quality color representation of this figure is available in the online issue.)
BMF EXPRESSION IN THE DIABETIC KIDNEY

DISCUSSION
The current study documents enhanced Bmf expression in RPTCs of db/db mice, STZ-induced diabetic mice, and kidneys from patients with T2DM and shows that Bmf overexpression enhances RPTC apoptosis, indicating a potential role for Bmf in mediating tubular atrophy in the diabetic kidney.

To identify the proapoptotic genes regulated by ROS in db/db mice RPTCs, we used gene chip microarrays as an initial screen (37). A combination of high P values, fold changes, and Gene Ontology annotation (32) allowed us to obtain an overview of the genetic regulation of RPTC apoptosis occurring in murine models of T2DM.

We identified Bmf as one of five putative proapoptotic genes that were differentially upregulated (P < 0.01 and >1.5-fold increase) in RPTs of db/db mice compared with db/m+ and db/db CAT-Tg mice. The upregulation of Bmf mRNA and protein expression in RPTCs of db/db mice was confirmed by real-time qPCR and immunostaining.

To confirm enhanced Bmf expression, we used an other model of diabetes, STZ-induced diabetes in mice (20). Our results document significant upregulation of Bmf expression in RPTCs of STZ-induced diabetes and its reversal by insulin.

There is evidence that HG induces apoptosis in RPTCs via ROS generation (20,38). Our present data show that HG also stimulates Bmf mRNA expression, which can be inhibited by rotenone, CAT, DPI, and apocynin. Cellular H2O2 and mitochondrial ROS levels were also significantly higher in RPTCs incubated in HG medium than in RPTCs cultured in normal glucose and were normalized in the presence of CAT (Supplementary Fig 2a and b). These observations indicate that ROS derived from mitochondrial oxidative metabolism may mediate, at least in part, HG-induced Bmf expression, which in turn would promote RPTC apoptosis.

To provide evidence that Bmf can directly induce RPTC apoptosis, we overexpressed Bmf in rat RPTCs. Transient transfection of RPTCs with rat Bmf cDNA resulted in activation of caspase-3 parallel with increases in the number of apoptotic cells, similar to that observed in transfected fibroblasts and cancer cells (39). We were unable to detect increases in endogenous rat Bmf expression in RPTCs by immunoblotting or immunofluorescence (data not shown) using the same anti-Bmf antibodies used for immunohistochemistry. Of note, Schmelzle et al. (40) also observed that commercially available anti-Bmf antibodies do not work in immunoblotting versus immunohistochemistry studies that show increased Bmf expression in RPTCs taken from nonmalignant portions of kidneys removed from diabetic patients due to kidney cancer compared with those taken from nondiabetic patients with kidney cancer. Interestingly, RPTCs overexpressing Bmf frequently stained positive for TUNEL in diabetic human kidney. Furthermore, the Affy 133plus chip assay revealed significantly upregulated Bmf expression (q < 0.01, where q value is a multiple testing corrected P value) in microdissected glomeruli (1.48-fold increase) and tubulo-interstitium (1.39-fold increase) from patients with T2DM compared with nondiabetic patients (kidney donors). The 1.39-fold versus 3-fold increases in Bmf expression in human diabetic tubulointerstitium and db/db mice RPTs, respectively, are difficult to reconcile. It is possible that Bmf mRNA expression in human tubulointerstitium might have been underestimated because of the use of a mixture of tubules and interstitium versus the >90% purity of our db/db mouse RPT fraction. Additional qPCR studies are needed to compare Bmf expression in human RPTs with or without T2DM.

Our results may have clinical implications for patients with T2DM. Because tubular apoptosis is one of the characteristic morphologic changes in human diabetic kidneys (15–17) and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology (2–4), we suggest that RPTC apoptosis may be an initial mechanism for tubular atrophy in T2DM. Our present data point toward Bmf as one of the mediators of this process. However, whether enhanced Bmf expression directly or indirectly induces RPTC apoptosis in human T2DM remains to be investigated.

In summary, the current study suggests an important role for Bmf in mediating RPTC apoptosis in the diabetic mouse kidney in vivo, and, likely, in diabetic human kidneys. Our observations raise the possibility that selective targeting of this proapoptotic protein may provide a novel approach in preventing or reversing the pathologic manifestations of DN, particularly tubular atrophy.

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