MafA-deficient and beta cell-specific MafK-overexpressing hybrid transgenic mice develop human-like severe diabetic nephropathy

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Abstract

Transcription factor MafA is a key molecule in insulin secretion and the development of pancreatic islets. Previously, we demonstrated that some of the MafA-deficient mice develop overt diabetes mellitus, and the phenotype of these mice seems to be mild probably because of redundant functions of other Maf proteins. In this study, we generated hybrid transgenic mice that were MafA-deficient and also overexpressed MafK specifically in beta cells ($MafA^{-/-}MafK^{+}$). $MafA^{-/-}MafK^{+}$ mice developed severe overt diabetes mellitus within 5 weeks old, and showed higher levels of proteinuria and serum creatinine. Histological analysis revealed that embryonic development of beta cells in the $MafA^{-/-}MafK^{+}$ mice was significantly suppressed and the reduced number of beta cells was responsible for the early onset of diabetes. Furthermore, after uninephrectomy, these mice demonstrated three characteristics of human diabetic nephropathy: diffuse, nodular, and exudative lesions. $MafA^{-/-}MafK^{+}$ mice might be a useful model for the analysis of human diabetic nephropathy.

Keywords

MafA, MafK, diabetes mellitus, diabetic nephropathy

Introduction

The transcription of genes that are involved in insulin production is regulated by a variety of transcription factors that are expressed specifically in pancreatic beta cells. It has been demonstrated that three conserved elements, A3, E1 and C1, which are found in the regulatory regions of the insulin genes, are indispensable for beta cell-specific insulin gene transcription [1-10]. Pancreatic duodenal homeobox factor-1 (PDX-1) binds to the A3 element and is involved in both insulin gene expression and pancreatic development [4,7,9]. The transcription factor BETA2 binds to E1 elements and also plays an important role in insulin production and islet development [6]. The C1 element is a critical *cis*-acting sequence for insulin gene expression, and is bound by the RIPE3b1 transcriptional activator. The gene that encodes the RIPE3b1 activator has been cloned and identified as MafA [8,10].

MafA is a member of Maf transcription factor family. Maf family proteins contain a C-terminal basic leucine zipper domain that binds to specific DNA sequences that are named Maf recognition elements (MAREs), and mediates dimerization of the proteins. Maf family proteins can be divided into two subgroups,

large and small Maf proteins, on the basis of presence or absence of an N-terminal activation domain. The members of the small Maf protein family, which include MafK, MafF, and MafG [11,12], do not possess the transactivation domain. These proteins can either act as transcriptional cofactors by forming heterodimers with members of the Cap 'n' collar (CNC) transcription factor family or as transcriptional repressors by binding to target MARE sequences as homodimers. The role that they play depends on the equilibrium concentrations of the CNC and small Maf proteins [13-16]. On the other hand, large Maf proteins contain an N-terminal activation domain and stimulate the transcription of target genes.

MafA is important for insulin gene transcription. Therefore, we generated MafA-deficient ($MafA^{-/-}$) mice and reported that these mice develop diabetes mellitus due to a decrease in insulin gene transcription, impaired glucose-stimulated insulin secretion and abnormalities of the pancreatic islets [17]. In spite of many previous reports that showed the importance of MafA for pancreatic beta cell function [2,8,18,19], the phenotype of $MafA^{-/-}$ mice seemed to be mild. This mild phenotype might be explained by the presence of other large Maf proteins, MafB and/or c-Maf,

that can activate the expression of insulin genes and other MafA target genes [3,8,18,20]. In addition to the generation of MafA$^{-/-}$ mice, we also generated transgenic mice that overexpressed MafK specifically in beta cells (MafK^{+}) mice [21]. MafK, which is a small Maf protein, acts as a dominant negative protein to suppress the effect of large Maf proteins in pancreatic beta cells. MafK^{+} mice demonstrated hyperglycemia at a young age, and then this phenotype disappeared. Furthermore, MafK^{+} mice did not develop overt diabetes. Detailed analysis of the mice revealed that up-regulation of endogenous MafA might compensate for the over-expression of MafK.

In this study, we generated MafA$^{-/-}$ mice that over-expressed MafK in pancreatic beta cells (MafA$^{-/-}$MafK^{+}**)** mice in order to depress MafA protein and transcriptional activity of other large Maf proteins in the beta cells, and evaluated whether the MafA$^{-/-}$MafK^{+} mice developed overt severe diabetes mellitus.

Materials and methods

Animals. MafA$^{-/-}$ mice [17] and MafK^{+} mice [21] were generated as described previously. All mice used were on an ICR genetic background. MafA$^{-/-}$ female mice were interbred with MafK^{+} male mice. The MafA$^{-/-}$MafK^{+} mice were obtained from interbreeding of MafA$^{+/-}$MafK^{+} and MafA$^{-/-}$ mice. Male mice were used in this study. Mice were fed with normal diet and maintained in specific pathogen-free conditions in a Laboratory Animal Resource Center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba, and the study was approved by the Institutional Review Board of the University.

Measurements of serum glucose and insulin. The serum concentration of glucose in fed animals and in animals subjected to a 12 hours overnight fast was measured using a Dry-chem 3500 automated analyzer for routine laboratory tests (Fuji Film, Inc., Tokyo, Japan). The serum insulin levels in fed animals were measured with a rat insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Analysis of urinary protein excretion and renal serological assays. The urine of each mouse was collected in an individual metabolic cage over a 24 hours period. The

amount of proteinuria was assessed by measuring the turbidity that was obtained with 3% sulphosalicylic acid. The concentrations of serum albumin, creatinine, blood urea nitrogen, and total cholesterol were measured using the Dry-chem 3500 analyzer.

Histopathological analysis. The pancreas and one of the kidneys from each mouse were fixed with 10% formalin and embedded in paraffin. Pancreatic sections were stained for histopathological examination with hematoxylin and eosin, and kidney sections were stained by the Periodic acid-Schiff (PAS). For semiquantitative histological analysis, the area of 30 glomerular tufts in each kidney section was measured by the Image J image processing program (National Institutes of Health, Bethesda, MD). The mean glomerular tuft was determined [22]. The other kidney from each mouse was snap-frozen and embedded in optimal cutting temperature compound for immunohistochemistry.

Immunohistochemistry. Pancreatic sections were immunostained with both anti-insulin antibody (Linco Research, St. Louis, MO) and anti-glucagon antibody (DAKO, Glostrup, Denmark) to investigate the distribution of alpha and beta cells.

Detection was performed using fluorescein secondary antibodies (Cortex Biochem, San Leandro, CA and ZYMED, San Francisco, CA). The frozen kidney sections were immunostained with anti-laminin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as the primary antibody and this was detected using the Histofine Kit (Nichirei, Tokyo, Japan).

Uninephrectomy of mice. To perform uninephrectomy of the mice, the left renal artery and ureter were ablated in eight-week-old mice under anesthesia. Twelve weeks after uninephrectomy, the mice were killed, the other kidney was removed and histological examination was carried out.

Statistical analysis. All results were expressed as means ± SEM. Multiple data comparisons were conducted using one-way analysis of variance (ANOVA) with the Bonferroni correction. Differences were considered statistically significant at P values that were less than 0.05.

Results

MafA$^{-/-}$MafK^{+} mice developed overt diabetes mellitus at 5 weeks of age

We first analyzed the blood glucose level of these mice. MafA$^{-/-}$MafK^{+} mice had already developed overt diabetes mellitus at 5 weeks of age (Table 1). The blood glucose levels of MafA$^{-/-}$MafK^{+} mice that had been fed were significantly higher than those of MafA$^{-/-}$ ($P < 0.01$), MafK^{+} ($P < 0.01$), or wild type ($P < 0.01$) mice at 5 weeks of age (Table 1). Even after fasting, the blood glucose levels in the MafA$^{-/-}$MafK^{+} mice were in excess of 500 mg/dl. Female MafA$^{-/-}$MafK^{+} mice displayed an almost identical phenotype to the male mice and hyperglycemia was detected during the observation period, which lasted for 30 weeks (data not shown). Serum insulin levels in the MafA$^{-/-}$MafK^{+} mice were lower than those in the wild type ($P < 0.01$), and MafK^{+} mice ($P < 0.01$). In addition to hyperglycemia, the MafA$^{-/-}$MafK^{+} mice showed growth retardation compared to wild type mice as their weight increased. We also evaluated the life span. Survival rate of MafA$^{-/-}$MafK^{+} mice (n=16) at 30 weeks was 37.5%, but those of the other groups were all 100% (wild type (n=15), MafK^{+} (n=8), and MafA$^{-/-}$mice (n=8)). MafA$^{-/-}$MafK^{+} mice had a short life span than the other

groups.

Morphological analysis of pancreatic islets of MafA$^{-/-}$MafK^{+} mice

The size of the pancreatic islets in 20-week-old MafA$^{-/-}$MafK^{+} mice was comparable with that of the wild type, MafK^{+}, and MafA$^{-/-}$mice (Fig. 1G, A, C and E). However, immunohistochemical analysis revealed that, although the mean total cell number for each islet was not significantly different between the wild type and MafA$^{-/-}$MafK^{+} mice, the islets in the MafA$^{-/-}$MafK^{+} mice were occupied by glucagon-positive cells (Fig. 1H). As we demonstrated previously, this pancreatic islet abnormality is one of the main phenotype of MafA$^{-/-}$ mice. In MafA$^{-/-}$MafK^{+} mice, the abnormal architecture of the islets, in particular the decrease in the number of beta cells and increase in the number of alpha cells, was more severe than in MafA$^{-/-}$ mice (Fig. 1H and F). This abnormal distribution of alpha and beta cells was observed from birth (Fig. 1J).

MafA$^{-/-}$MafK^{+} mice exhibit diabetic nephropathy-like phenotype upon biochemical examination

Due to the fact that MafA$^{-/-}$MafK^{+} mice developed severe diabetes mellitus at an early age and survived without insulin treatment, we thought that it might be possible to use these mice as a model for diabetic nephropathy. Predictably, these mice demonstrated polyuria, and displayed increased proteinuria, serum total cholesterol and blood urea nitrogen as compared with wild type mice (Table 2). This phenotype is similar to the nephrotic syndrome observed in human diabetic nephropathy. Moreover, there was a significant elevation of the serum creatinine level in MafA$^{-/-}$MafK^{+} mice.

Histological analysis of the kidneys of MafA$^{-/-}$MafK^{+} mice

Next, we performed histological analysis of kidneys from 20-week-old MafA$^{-/-}$MafK^{+} mice. Glomerular hypertrophy and mesangial matrix expansion, which are the most characteristic lesions of diabetic nephropathy [22] were observed in PAS-stained kidney sections from MafA$^{-/-}$MafK^{+} mice (Fig. 2G). In fact, the glomerular surface area in the sections from MafA$^{-/-}$MafK^{+} mice was significantly larger than in the sections from wild type mice as determined by quantitative analysis

(Fig. 3). The ratio of the mean glomerular surface area of MafA$^{-/-}$MafK^{+} mice to that of wild mice was 1.79 ± 0.09. Furthermore, the kidney weight to body weight ratio of the MafA$^{-/-}$MafK^{+} mice was also significantly heavier than those of the wild type mice (Table 2). To analyze mesangial matrix expansion, we performed immunohistochemical staining of laminin, since laminin is a component of the mesangial matrix [22]. We detected strong anti-laminin staining and confirmed that glomerular matrix expansion had occurred in the MafA$^{-/-}$MafK^{+} mice (Fig. 2H).

Uninephrectomy of MafA$^{-/-}$MafK^{+} mice caused the nodular-like lesions that are observed in human diabetic nephropathy

To increase the load on a single glomerulus, we performed uninephrectomy in 8-week-old wild type, MafK^{+}, MafA$^{-/-}$ and MafA$^{-/-}$MafK^{+} mice. Twelve weeks after uninephrectomy, only MafA$^{-/-}$MafK^{+} mice showed impairment of renal function upon serological testing (Table 3), and histological examination. MafA$^{-/-}$MafK^{+} mice after uninephrectomy demonstrated a more severe mesangial matrix expansion than that of MafA$^{-/-}$MafK^{+} mice (Fig. 4D). On the other hand, MafA$^{-/-}$, MafK^{+}, and wild

type mice showed no change after uninephrectomy (Fig. 4 A,B,C). Moreover, the kidneys of the MafA$^{-/-}$MafK^{+} mice contained diffuse lesions characterized by a widespread increase in periodic acid-Schiff-positive material within the mesangium in textbook [23], nodular lesions, and exudative lesions, all of which are observed in severe human diabetic nephropathy (Fig. 4 E,F,G).

Discussion

MafA$^{-/-}$MafK^{+} mice displayed a more severe phenotype than the MafA$^{-/-}$ mice, and developed diabetes mellitus at a younger age. Histological analysis demonstrated that the pancreatic islets of MafA$^{-/-}$MafK^{+} mice were almost completely occupied by glucagon-producing alpha cells. This phenotype could be explained by the results of recent reports and our own preliminary observations. Nishimura *et al.* and Artner *et al.* have demonstrated that MafB is expressed in both alpha and beta cells during embryonic development and that MafB deficiency induces a reduction in the mass of both alpha and beta cells [24,25]. Due to the fact that we demonstrated previously that the number of alpha and beta cells in new born MafA$^{-/-}$ mice is not abnormal [17], this suggested that MafB is a key large Maf factor in the development of alpha and beta cells during embryogenesis. This idea is also supported by our preliminary observation that MafA$^{-/-}$MafB$^{-/-}$ embryos display an almost same reduction in both alpha and beta cells as that seen in MafB$^{-/-}$ embryos at 18.5 days (data not shown). MafK, which acts as dominant negative protein to inhibit the activity of large Maf proteins, may suppress MafB function specifically in beta cells,

thus inducing a phenotype that resembles that of the MafB-deficient embryos. However, MafK is not expressed in the alpha cells and would not interfere with MafB function in these cells. Therefore, alpha cell expansion would be induced in the embryos.

As we have demonstrated in this paper, MafA$^{-/-}$MafK^{+} mice have the potential to become a new model for diabetic nephropathy. These mice developed diabetes mellitus before 5 weeks of age and began to demonstrate polyuria at this time. In the biochemical analysis, increased creatinine, blood urea nitrogen, total cholesterol and proteinuria excretion were observed in the MafA$^{-/-}$MafK^{+} mice. The histological appearance of kidney sections from the MafA$^{-/-}$MafK^{+} mice showed more severe glomerular hypertrophy and mesangial matrix expansion than in those from wild type, MafK^{+} and MafA$^{-/-}$ mice. These data suggested that the renal phenotype of our hybrid transgenic mice is similar to human diabetic nephropathy.

Human diabetes mellitus is classified into four types: type 1, type 2, other specific types and gestational diabetes [26]. Type 1 diabetes mellitus is characterized by the destruction of beta cell due to an autoimmune process, and this usually leads to

insulin deficiency. Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissues and insufficient insulin secretion by the beta cells. This is highly associated with a family history of diabetes, older age, obesity and lack of exercise. The mouse models of diabetes are also classified as type 1 or type 2 diabetes mellitus [27]. $MafA^{-/-}MafK^{+}$ mice developed diabetes at an early age because of inadequate embryonic development of beta cells, but did not show autoimmune pancreatitis or obesity after birth. In this sense, it is difficult to classify $MafA^{-/-}MafK^{+}$ mice simply as a model of type 1, or 2, diabetes, although the $MafA^{-/-}MafK^{+}$ mouse is a good animal model for hyperglycemia.

The major deficiency in animal models of diabetic nephropathy is the absence of severe human-like diabetic histopathological findings [27]. The db/db mouse is one of the most well known models of diabetic nephropathy [28]. This mouse shows hyperglycemia at 8 weeks of age and after 4-6 weeks of hyperglycemia, mesangial matrix expansion is observed. The level of mesangial matrix expansion and glomerular hypertrophy in the db/db mice is mild compared to that in the $MafA^{-/-}MafK^{+}$ mouse. In addition, most mouse models of diabetes show

gender-specific differences in their phenotype and the appearance of nodular lesions in these mice is rare [22]. However, in the MafA$^{-/-}$MafK^{+} mouse, both male and female mice developed diabetes mellitus at 5 weeks of age, and hyperglycemia persisted throughout the observation period. Furthermore, after uninephrectomy, nodular lesions, which are the representative histological feature of human diabetic nephropathy, were found in our mice. In the mouse models, diffuse lesions, nodular lesions and exudative lesions are generally absent and we could not detect nodular lesions in 20-week-old MafA$^{-/-}$MafK^{+} mice [27]. Uninephrectomy has been used experimentally to enhance the development of diabetic nephropathy [29,30]. MafA$^{-/-}$MafK^{+} mice demonstrated three characteristics of human diabetic nephropathy after uninephrectomy: diffuse lesions, nodular lesions, and exudative lesions. From these results, we conclude that MafA$^{-/-}$MafK^{+} mice might provide a new mouse model for diabetes and useful tool for analyzing the pathogenesis of human diabetic nephropathy.

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Figures legends

Fig. 1. Histopathological examination of pancreatic islets. Hematoxylin and eosin staining of pancreatic islets in 20-week-old wild type (A), MafK^{+} (C), MafA$^{-/-}$ (E) and MafA$^{-/-}$MafK^{+} mice (G). Magnification is x 50 and each bar represents 100 μm. Immunohistochemistry of pancreatic islets. Immunofluorescence staining of insulin (red) and glucagon (green) in 20-week-old wild type (B), MafK^{+} (D), MafA$^{-/-}$ (F) and MafA$^{-/-}$MafK^{+} mice (H). Magnification is x 100 and each bar represents 100 μm. Immunofluorescence staining of insulin (red) and glucagon (green) in wild type (I), and MafA$^{-/-}$MafK^{+} mice (J) on postnatal day 1. Magnification is x 200 and each bar represents 20 μm.

Fig. 2. Histological examination of the kidney. Periodic acid-Schiff staining in 20-week-old wild type (A), MafK^{+} (C), MafA$^{-/-}$ (E) and MafA$^{-/-}$MafK^{+} mice (G). Magnification is x 200 and each bar represents 100 μm. Immunohistochemical staining of laminin in 20-week-old wild type (B), MafK^{+} (D), MafA$^{-/-}$ (F) and MafA$^{-/-}$MafK^{+} mice (H). Magnification is x 350 and each bar represents 100 μm.

Fig. 3. Semiquantitative histological analysis of glomerular surface area in 20-week-old wild type (n = 5), MafK^{+} (n = 5), MafA$^{-/-}$ (n = 5) and MafA$^{-/-}$MafK^{+} (n = 5) mice. The ratios of the mean glomerular tufts of MafA$^{-/-}$MafK^{+} mice were significantly larger than that of MafK^{+}, MafA$^{-/-}$ and wild type mice. *P < 0.01.

Fig. 4. Histological examination of wild type (A), MafK^{+} (B), MafA$^{-/-}$ (C) and MafA$^{-/-}$MafK^{+} (D) mice at 12 weeks after uninephrectomy. Magnification is x 200. Diffuse lesions are observed at a magnification of x 600 (E), exudative lesions at a magnification of x 600 (F), and nodular lesions at a magnification of x 600 (G). The arrowhead shows an exudative lesion, and the arrows show nodular lesions. Periodic acid-Schiff staining. Each bar represents 100 μm.

Table 1 Biochemical data of the experimental mice.

	Wild	MafK^{+}	MafA$^{-/-}$	MafA$^{-/-}$MafK^{+}
n	8	7	7	6
Serum				
Glucose (5W) (mg/dl)	175.7 ± 8.1	226.7 ± 5.3	217.6 ± 10.5	900.8 ± 84.6*
Glucose (10W) (mg/dl)	173.0 ± 8.2	193.6 ± 11.1	167.6 ± 8.8	999.4 ± 104.2*
Glucose (20W) (mg/dl)	159.8 ± 38.3	158.0 ± 21.6	252.3 ± 35.2	853.3 ± 64.1*
Insulin (10W) (ng/ml)	0.93 ± 0.09	1.49 ± 0.20*	0.66 ± 0.2	0.28 ± 0.10*
Body weight (5W) (g)	25.2 ± 1.0	24.9 ± 0.5	26.4 ± 0.4	23.0 ± 0.6
Body weight (10W) (g)	35.1 ± 0.5	32.2 ± 0.8*	30.8 ± 1.1*	25.8 ± 1.5*
Body weight (20W) (g)	40.8 ± 0.8	34.3 ± 0.8*	38.7 ± 0.7	27.9 ± 1.0*

Values are expressed as means ± SEM. Wild, wild type.

*P< 0.01 compared with Wild mice.

Table 2 Biochemical data of the experimental mice

	Wild	MafK^{+}	MafA$^{-/-}$	MafA$^{-/-}$MafK^{+}
n	7	6	5	5
Serum				
ALB (10W) (g/dl)	2.2 ± 0.0	2.2 ± 0.0	2.4 ± 0.1	1.9 ± 0.1**
ALB (20W) (g/dl)	2.6 ± 0.1	2.1 ± 0.1*	2.2 ± 0.1**	1.8 ± 0.1*
CRE (10W) (mg/dl)	0.35 ± 0.01	0.34 ± 0.02	0.38 ± 0.02	0.42 ± 0.02*
CRE (20W) (mg/dl)	0.34 ± 0.04	0.37 ± 0.06	0.41 ± 0.03	0.48 ± 0.04**
BUN (10W) (mg/dl)	20.6 ± 1.1	23.4 ± 0.9	24.3 ± 3.1	39.6 ± 3.5*
BUN (20W) (mg/dl)	26.3 ± 3.5	30.9 ± 6.7	27.0 ± 3.0	45.0 ± 7.8**
T-CHO (10W) (mg/dl)	155.2 ± 4.5	104.0 ± 7.7**	143.0 ± 30.3	207.8 ± 23.8**
T-CHO (20W) (mg/dl)	124.0 ± 6.1	98.3 ± 19.3	142.5 ± 10.6	260.0 ± 30.5*
Urinary				
Protein (10W) (mg/day)	3.5 ± 0.6	3.7 ± 1.0	4.4 ± 1.7	10.8 ± 2.4*
Protein (20W) (mg/day)	4.9 ± 1.6	5.7 ± 0.8	3.9 ± 1.8	8.8 ± 2.3
Kidney/BW (20W) (%)	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.9 ± 0.3*

Values are expressed as means ± SEM. Wild, wild type. ALB, albumin; CRE, creatinine; BUN, blood urea nitrogen; T-CHO, total cholesterol; BW, body weight. Kidney, the mean weight of kidneys.

*$P < 0.01$ compared with Wild mice.

**$P < 0.05$ compared with Wild mice.

Table 3 Biochemical data after uninephrectomy

	Wild	MafK^{+}	MafA$^{-/-}$	MafA$^{-/-}$MafK^{+}
n	3	5	3	3
Serum				
Glucose (20W) (mg/dl)	164.0 ± 14.4	210.6 ± 13.4	173.0 ± 19.7	1033.0 ± 55.9*
CRE (20W) (mg/dl)	0.37 ± 0.03	0.37 ± 0.02	0.35 ± 0.06	0.52 ± 0.04**
BUN (20W) (mg/dl)	32.0 ± 1.5	35.8 ± 2.7	33.3 ± 2.2	53.4 ± 7.0*
Urinary				
Protein (20W) (mg/day)	1.9 ± 0.5	2.7 ± 0.6	1.7 ± 0.3	4.2 ± 0.3*
Kidney/BW (20W) (%)	1.1 ± 0.0	0.9 ± 0.1	1.1 ± 0.1	2.4 ± 0.3*

Values are expressed as means±SEM. Wild, wild type. CRE, creatinine; BUN, blood urea nitrogen; BW, body weight. Kidney, a single kidney weight.

*$P < 0.01$ compared with Wild mice.

**$P < 0.05$ compared with Wild mice.

Fig. 1.

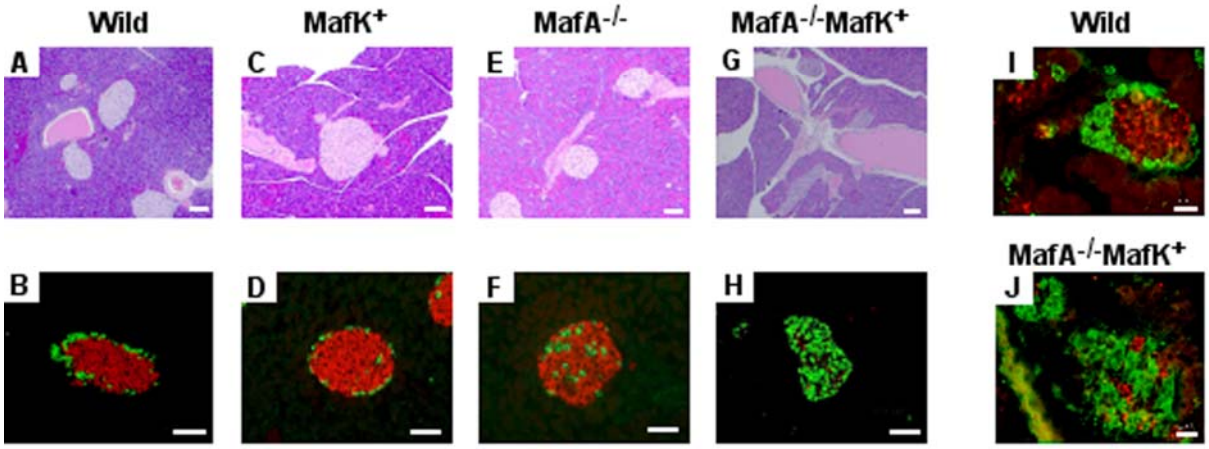


Fig. 2.

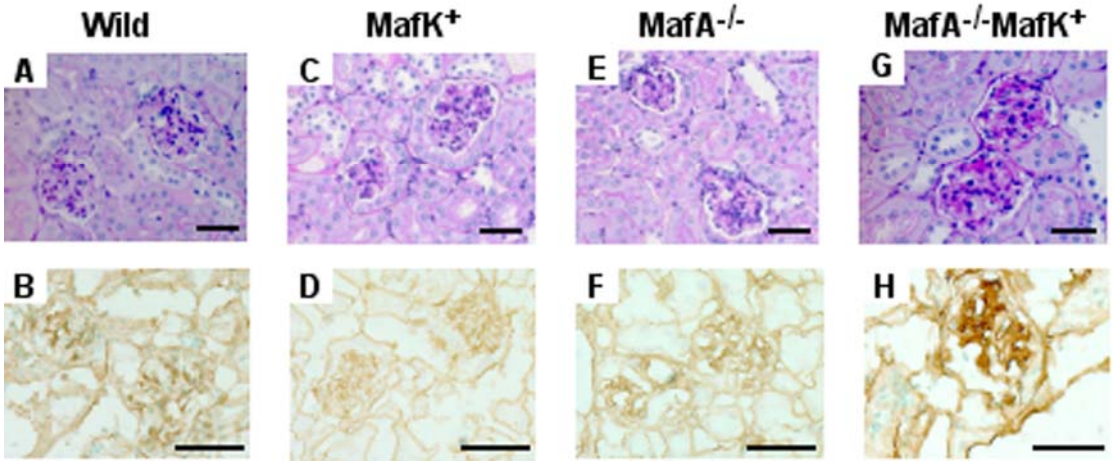


Fig. 3.

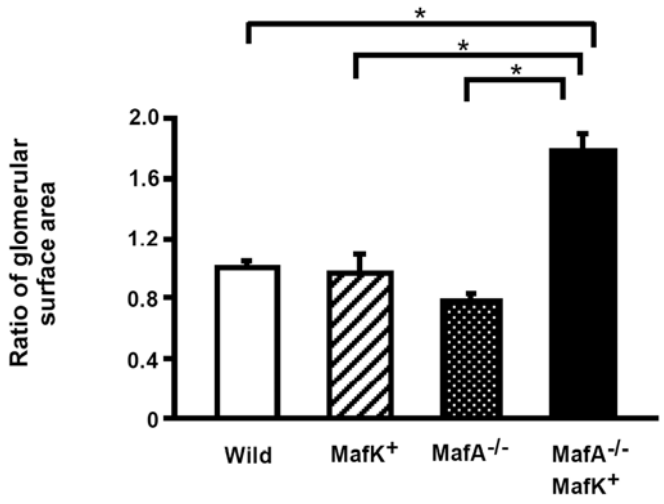
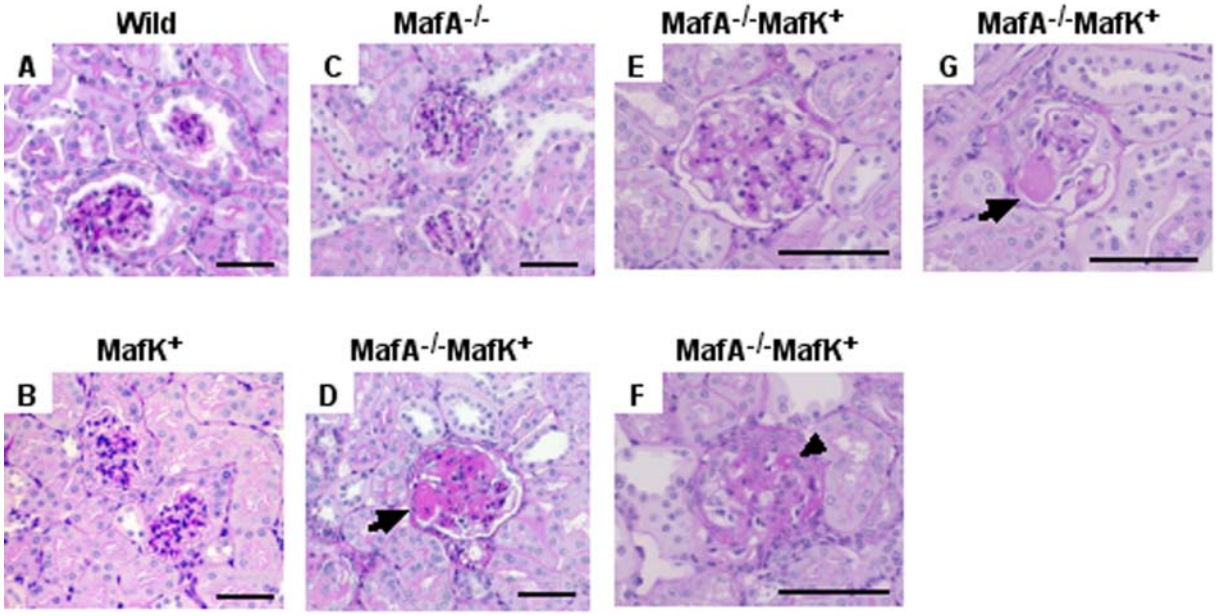

*
*
*
Ratio of glomerular surface area
2.0
1.6
1.2
0.8
0.4
0
Wild
MafK+
MafA-/-
MafA-/- MafK+

Fig. 4.


Wild
A
MafA-/-
C
MafA-/-MafK+
E
MafA-/-MafK+
G
MafK+
B
MafA-/-MafK+
D
MafA-/-MafK+
F