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Medaka fish, *Oryzias latipes*, as a model for human obesity-related glomerulopathy

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ABSTRACT

Obesity, an ongoing significant public health problem, is a part of complex disease characterized as metabolic syndrome. Medaka and zebrafish are useful aquatic experimental animals widely used in the field of toxicology and environmental health sciences and as a human disease models. In medaka, simple feeding of a high fat diet (HFD) can induce body weight gain, excessive accumulation of visceral adipose tissue, hyperglycemia, hyperlipidemia, and steatohepatitis, which mimics human metabolic syndrome. In the present study, to explore the possibility that the adult medaka fed with HFD (HFD-medaka) can be used as an animal model for human metabolic syndrome-associated glomerular disease, including obesity-related glomerulopathy (ORG), we analyzed structural alterations and protein expression in the mesonephric kidney of HFD-medaka. We found that the histopathology was consistent with glomerulomegaly accompanied by the dilation of glomerular capillaries and proliferative expansion of the mesangium, a condition partially comparable to human ORG. Moreover, expressions of several kinds of kidney disease-related proteins (such as MYH9, SM22α) were significantly elevated. Thus, the HFD-medaka has a high potential as an animal model useful for exploring the mechanism underling human ORG.

1. Introduction

Medaka, *Oryzias latipes*, is a freshwater fish native to East Asian countries, primarily Japan, Korea, China, and Taiwan. This small fish is one of the most useful aquatic experimental animals due to its completely sequenced genome, high fecundity, transparency of embryos, and adaptation to a wide range of temperatures [1]. As in zebrafish, researchers can manipulate gene expression in medaka by the use of forward and reverse genetic techniques, and medaka is also widely used in the fields of developmental biology, toxicology, and environmental health sciences [1–3].

Recently, it was reported that, in adult medaka, simple feeding of a high fat diet (HFD) could induce body weight gain, excess accumulation of visceral adipose tissue, hyperglycemia and hyperlipidemia (both hypertriglyceridemia and hypercholesterolemia) over a relatively short period. In this condition, medaka adult that are fed HFD (HFD-medaka) consistently exhibit a fatty liver and subsequently develop non-alcoholic steatohepatitis (NASH) [4]. Using this system, several useful drugs for NASH were found [4–6]. In humans, non-alcoholic fat liver disease followed by NASH initially cause chronic liver dysfunction that may later progress to liver cirrhosis and ultimately hepatocellular carcinoma, and thus new guideline of NASH was developed [7].

Obesity and weight control are ongoing important public health problems. Broadly viewed, obesity may be considered part of a complex disease characterized as obesity-initiated metabolic syndrome, which comprises abdominal obesity, hypertriglyceridemia, low HDL cholesterol, hypertension, and hyperglycemia [8,9]. The HFD-medaka adult exhibits a variety of similar features consistent with those characteristic seen in human metabolic syndrome, and therefore can be utilized as an informative animal model for the disease. Metabolic syndrome is associated with increased prevalence of chronic kidney disease (CKD), and obesity itself is also known to induce obesity-related glomerulopathy (ORG), which is defined by a combination of obesity, glomerular enlargement (glomerulomegaly), and

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proteinuria with or without nephrotic state [10–13]. Kambham and colleagues (2001) reported that ORG patients were all associated with glomerulomegaly [14]. Two histological types were identified: glomerulomegaly with or without focal segmental glomerulosclerosis (FSGS), a pathological feature characterized by partial scarring of the glomerular structure.

In the present study, to explore the potential of the HFD-medaka as an animal model for human metabolic syndrome-associated glomerular disease including ORG, we analyzed the histopathology and protein expression in the mesonephric kidney of HFD-medaka. We found these fish demonstrated characteristic histopathology partially compatible to human ORG. Moreover, the expression of several kidney-disease-related proteins were significantly elevated. We thus present the HFD-medaka adult as a new animal model to study human ORG.

2. Materials and methods

2.1. Fish maintenance

Medaka (Cab strain) were maintained and raised at 28.5 °C under a 14-h light/10-h dark cycle. Each tank was supplied daily with 200 mg of food, which was consumed within 14 h. The energy content of the control diet (Hikari Crest; Kyorin, Hyogo, Japan) was 3.3 kcal/g with 25.3% from fat, 62.5% from protein and 3.8% from carbohydrates. The energy content of the HFD (HFD32; CLEA Japan, Tokyo, Japan) was 5.1 kcal/g with 56.7% from fat, 20.1% from protein and 23.2% from carbohydrates [4]. Experiments were covered by protocols approved from the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (IACUC protocol no. 12-033).

2.2. Blood glucose level

Measurement of blood glucose level was performed as previously reported [4]. In brief, blood samples were obtained from medaka at 4, 8, and 12 weeks after the beginning of HFD feeding. Fish were anesthetized with tricaine and then bled by cutting a ventral portion of the tail fin. Blood glucose levels were determined using a Contour blood glucose monitoring system (Bayer HealthCare).

2.3. Histological analysis

Mesonephric kidneys were fixed with histology fixative (1.5% glutaraldehyde, 4% paraformaldehyde, 3% sucrose in 0.1 M phosphate buffer) for hematoxylin and eosin (HE) staining, and with 4% paraformaldehyde/PBS for periodic acid-Schiff (PAS) and periodic acid–methenamine–silver (PAM) staining. Fixed samples were dehydrated and embedded in JB-4 resin (Polysciences). Four micron sections were stained with HE (BBC Biochemical), Accustain Silver Stain kit, and PAS Staining System (Sigma–Aldrich). Transmission electron microscopy was conducted as previously described [15] and presented in Supplemental material S1.

2.4. Proteomic analysis

Proteomic analysis for kidney protein was conducted as presented in Supplemental material S2.

3. Results

3.1. Blood glucose level in the HFD-medaka

To confirm an alteration of metabolic condition in the HFD-medaka, we examined blood glucose level. Diet manipulation was initiated in medaka adult at 8 weeks of age. In control medaka, blood glucose levels measured at 4, 8, and 12 weeks later returned values of 105 ± 7, 90 ± 8, and 92 ± 18 mg/dl, respectively (n = 10 in each group) (Fig. 1A). In HFD-medaka, blood glucose level was elevated already at 4 weeks after diet initiation (160 ± 74 mg/dl), and sustained at 8 and 12 weeks (177 ± 62, and 173 ± 58 mg/dl, respectively) (n = 10 in each group) (Fig. 1A). At all the timepoints examined, blood glucose levels were higher in the HFD-medaka compared to those of the control with statistical significance.

3.2. Glomerular histopathology in the HFD-medaka

First, we examined the normal structure of the mesonephric glomerulus in control medaka. The glomeruli were frequently found beneath the renal capsule, which consisted of fine connective tissue (Fig. 1B1–B3). Like mammals, each glomerulus exhibited a well-developed glomerular capillary (Fig. 1B1–B3) and an arborized mesangium (Figs. 2A1–A3 and 3A) in medaka adult. Glomeruli were smaller in medaka (45–65 μm in diameter) than those reported in mouse (73 μm), rat (122 μm), and human (201 μm) [16].

In HFD medaka, prominent glomerular enlargement (120–200 μm in diameter) was recognized at all the timepoints examined and was associated with mesangial expansion and glomerular capillary dilation (Fig. 1C1–C3). In the expanded mesangium, cellularity was increased and PAS-positive mesangial matrix was prominent (Fig. 2B1–B3). However, nodular lesions, defined as characteristic structures of amorphous appearance in human diabetic nephropathy [17], were not recognized, as far as we observed. At 8 and 12 weeks, the glomeruli which closely resembled the lesion called as mesangiolysis in several kinds of human and experimental glomerulonephritis [18], were frequently observed (Figs. 1C3, 2B3 and 3D).

To further investigate the glomerular histopathology, we performed transmission electron microscopy. In control medaka kidney, the glomerular capillary wall was thin and consisted of podocyte foot processes, GBM, and fenestrated endothelial cells, as observed in mammalian metanephric glomerulus (Fig. 3B). In HFD-medaka, the glomerular capillary wall displayed an enlarged subendothelial space, which is a newly formed interspace between the GBM and endothelial cells (Fig. 3E). In the space, we often found mesangial cell processes, which was reminiscent of mesangial interposition found in human membranoproliferative glomerulonephritis [19]. At 12 weeks HFD, mesangiolysis was frequently found in the expanded mesangium (Fig. 3F).

3.3. Alterations of protein expression in the kidney of HFD-medaka

To identify proteins that are involved in the glomerulopathy, we conducted a proteomic analysis of proteins extracted from whole mesonephric kidney of HFD-medaka at the early phases (1, 2, 3, and 4 weeks after the beginning of HFD). We identified 927 classes of protein common to all of the timepoints by HPLC-Electrospray Ionization-MS/MS assays followed by database searches. Among these proteins, 18 proteins were up-regulated and 2 proteins were markedly down-regulated in the HFD-medaka, in comparison with control (0 week, prior to the HFD), as shown in Table 1.

4. Discussion

The HFD-medaka mimics several features of the human metabolic syndrome, which is characterized by a combination of abdominal obesity, hypertriglyceridemia, low level of HDL cholesterol, hypertension, and hyperglycemia. All of these components except for hypertension were recognized in the HFD-medaka, as

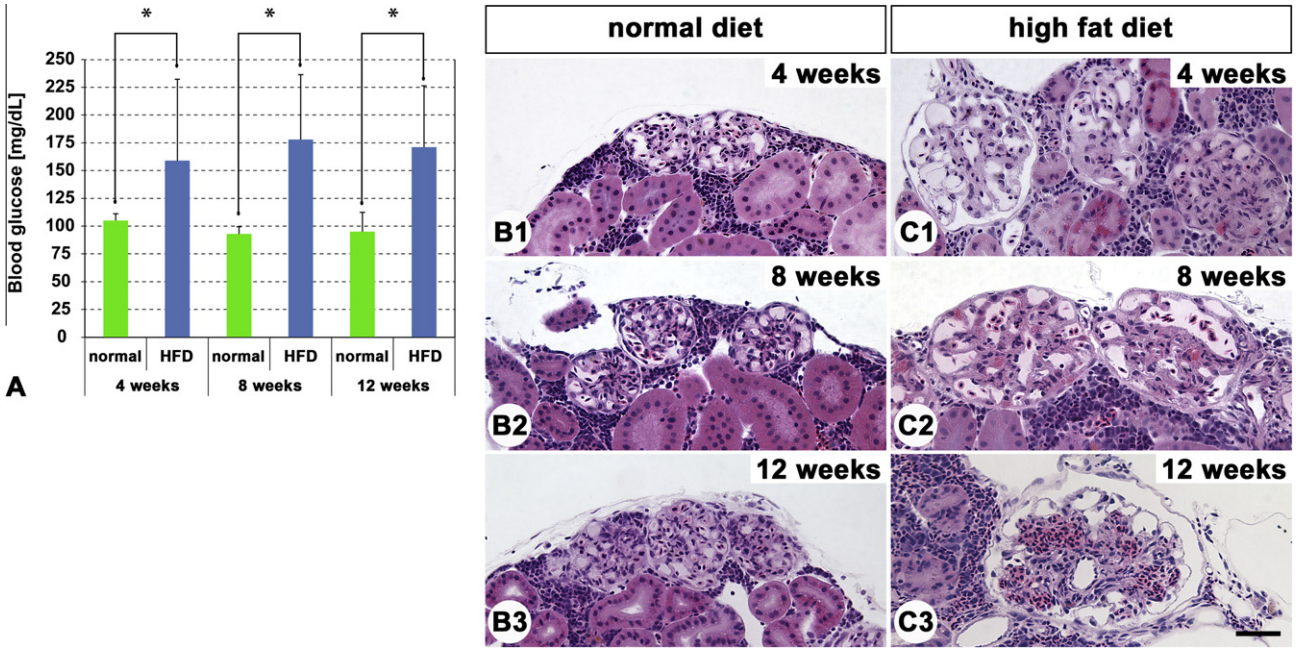

Fig. 1. Elevated blood glucose levels and glomerulomegaly in HFD-medaka. (A) In HFD-medaka, blood glucose levels were elevated with statistical significance already at 4 weeks after HFD initiation, and highly sustained at 8 and 12 weeks, compared with control. $n = 10$ in each group. $^{*}p < 0.05$. (B1–B3) Glomeruli in the control medaka. HE-stained sections show overall features of the glomerular structure. Within the glomeruli, well-developed fine capillaries are recognized. (C1–C3) Glomeruli in the HFD-medaka. Prominent glomerular enlargement, which is associated with capillary dilatation and mesangial expansion, is observed as early as at 4 weeks after HFD initiation (C1). Bar scale, 50 μm.

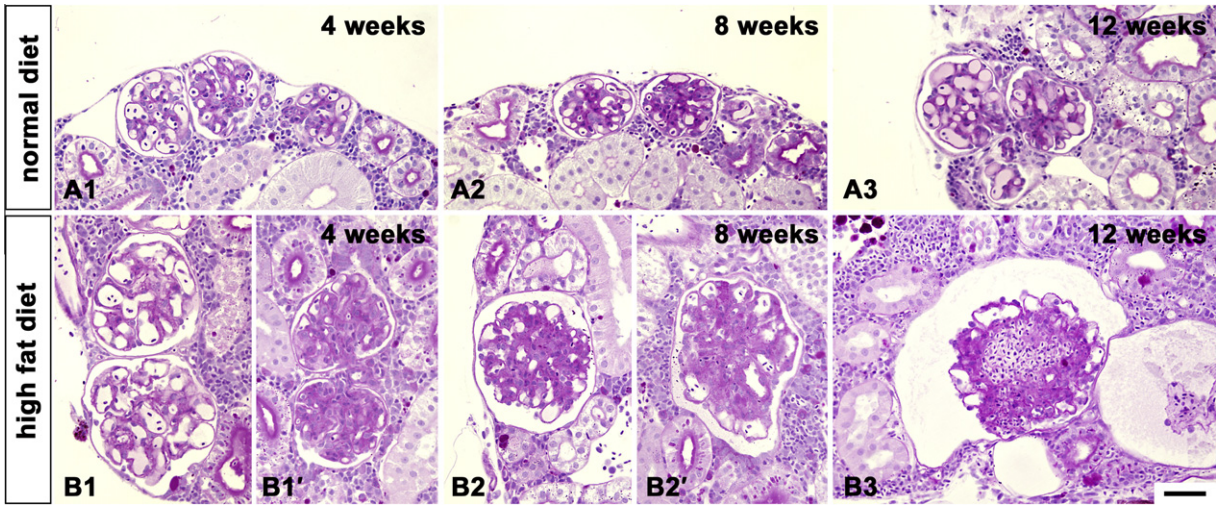

Fig. 2. Mesangial proliferation in HFD-medaka glomeruli. PAS-stained sections denote the structural alteration of mesangium. (A1–A3) Glomeruli in the control medaka. Within glomeruli, PAS-positive mesangial matrix is arborized in shape. (B1–B3) Glomeruli in the HFD-medaka. Glomerular enlargement with mesangial matrix expansion is already observed at 4 weeks (B1, B1′). At 8 weeks, PAS-positive matrix is massively accumulated in the expanded mesangial area (B2, B2′). Within the mesangial area are found numerous nuclei representing proliferated mesangial cells, which are counter-stained by hematoxylin (faint blue in B2, B2′). At 12 weeks, within the expanded mesangial area, a region with faint PAS-positive matrix is frequently found (B3). Bar scale, 50 μm.

previously reported [4–6]. Several kinds of glomerular disease can be induced in metabolic syndrome patients, including diabetes nephropathy, FSGS, and ORG. In the present study, the HFD-medaka consistently displayed excess accumulation of visceral adipose tissue and glomerulomegaly without diabetic lesions and FSGS, indicating that the HFD-medaka mimics human ORG, which is characterized by obesity, proteinuria, and glomerulomegaly [11].

4.1. Glomerulopathy in HFD-medaka in comparison with human ORG

In 1974, an association between massive obesity and nephrotic proteinuria was first reported [12]. Since then, the development of glomerulomegaly and FSGS has been linked to massive obesity [20–22], although most of these associations have been limited to case reports or small autopsy series. Kambham and colleagues (2001) reported the first large-scale study defining the clinical and pathological features of ORG that exhibit a combination of obesity, proteinuria, and glomerulomegaly, and some populations further develop a secondary FSGS [14].

In the HFD-medaka, FSGS was not recognized at all, as far as we observed. We consider the difference of blood (intraglomerular) pressure between teleost fish and mammals could cause such discrepancy, as speculated below. In mammals, average blood pressure is maintained around 100 mm Hg and high intraglomerular

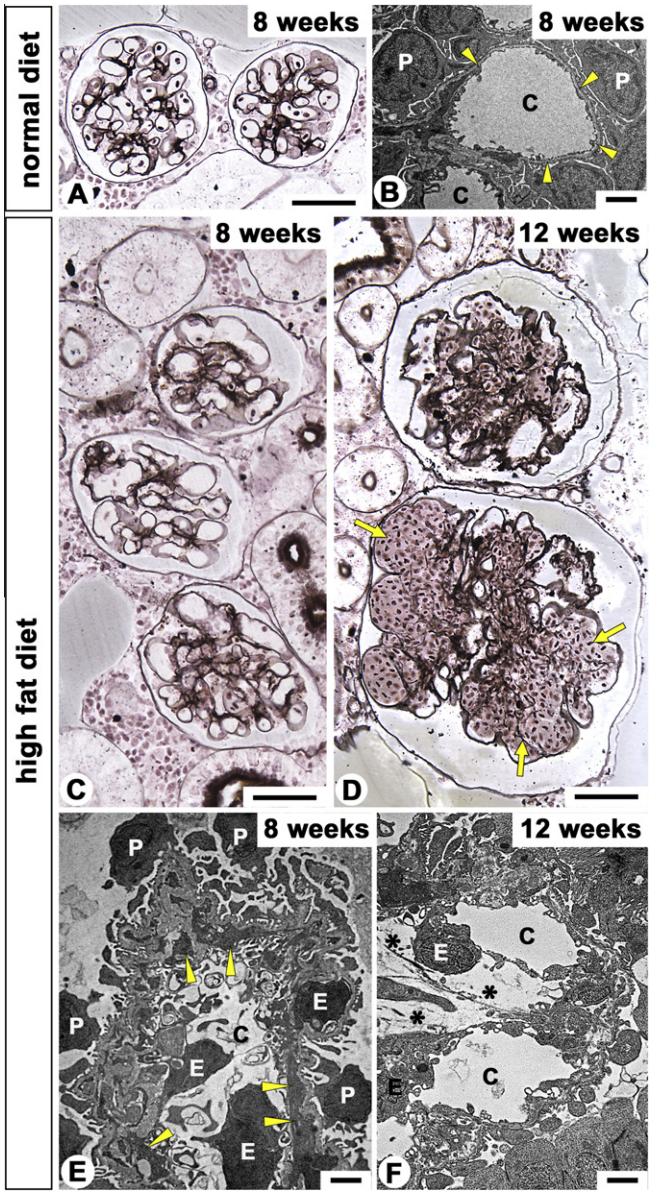

Fig. 3. Mesangiolysis and mesangial interposition in HFD-medaka glomeruli. (A and B) Glomeruli in the control medaka (8 weeks after HFD initiation). PAM-stained sections visualize mesangial matrix, which is arborized in shape (A). Capillary diameter is fairly uniform throughout the glomerulus (A). Transmission electron micrograph shows the glomerular capillary wall (arrowheads) is thin and consists of podocyte foot processes, GBM, and fenestrated endothelial cells (B). (C–F) Glomeruli in the HFD-medaka. Glomerular capillaries already vary in size from small to large at 8 weeks after HFD initiation (C). Mesangiolysis, which is characterized as an extremely expanded mesangial region containing low amounts of PAM-positive mesangial matrix, is frequently found at 12 weeks (arrows in D). Glomerular capillary wall become extremely thickened and form a subendothelial space, defined as a newly formed interspace between the GBM and endothelial cells. In the space, mesangial cell processes are frequently found (arrowheads in E). Mesangiolysis is found in the expanded mesangial region (asterisks in F). C, capillary lumen; E, glomerular endothelial cell; P, podocyte cell body. Bar scales, 50 μm in A, C and D; 1 μm in B, E and F.

capillary pressure is consistently maintained to effectively promote a substantial degree of glomerular filtration. To mechanically protect the glomerular structure against the higher pressure, the actin cytoskeleton (actin bundle) is well-developed within the podocyte foot processes in mammals [23]. However, in spite of the protective apparatus, structural breakdown of the glomerulus and followed by FSGS are induced by mechanical stress such as hypertension. On the other hand, in adult zebrafish, the peak systolic blood pressure is only 1.51 ± 0.38 mm Hg at the ventral aorta [24], and it is reasonable that medaka exhibit similar magnitude of pressure, although this has not been examined so far. Thus the mechanical stress to the glomerular wall would be far less in small teleost fish than in mammals, which may explain the putative absence of FSGS in the HFD-medaka.

4.2. HFD-medaka as a model for human ORG

Medaka is widely used as an animal model for toxicological testing for carcinogens and endocrine disruptors, for drug screening, and for human diseases [1–3]. In the HFD-medaka, the characteristic metabolic alteration and glomerulopathy developed after a relatively short period (merely 4 weeks after the beginning of HFD-feeding), represents a benefit for rapid screening of drugs proposed to prevent or ameliorate HFD-induced glomerulopathy.

Angiotensin converting enzyme inhibitor (ACEI) and/or angiotensin type II receptor blocker (ARB) therapy have beneficial effects aimed at preferentially reducing the efferent (postglomerular) arteriole resistance and subsequently intraglomerular pressure. Alternatively, these therapies may act directly on glomerular mesangial cells to prevent their proliferation and production of excess matrix material [25,26]. The application of ACEI and/or ARB in ORG also seems rational, although there is no long-term data describing the effects of these drug treatments on ORG. The renin–angiotensin–aldosterone system is recognized from teleost fish to mammals [27,28]. Indeed, we previously identified the expression of *renin* mRNA in the interglomerular mesangial area between the pronephric glomerulus [29]. A logical next experiment would be to examine whether ACEI and/or ARB are capable of preventing or ameliorating the glomerulopathy in HFD-medaka, and to further establish a screening system for novel drugs effective in metabolic syndrome-associated glomerular disease using the fish model.

4.3. Proteins related to the glomerulopathy in the HFD-medaka

Proteomic analysis revealed that several kinds of cytoskeletal proteins (MYH9, SM22α, radixin, filamin-A, and cytoskeletal tropomyosin) were up-regulated in the kidney of HFD-medaka. The elevated expressions of these proteins is highly likely to be involved in the dynamic structural alteration of the stressed glomeruli, other renal parenchyma, and interstitium.

MYH9, also known as non-muscle myosin heavy chain IIA, is localized in the cell cortex and stress fiber in non-migrating cells [30]. Arrondel and collegues (2001) reported MYH9 was immunohistochemically detected in the glomeruli, and in arteriolar and peritubular capillary endothelial cells in human kidney [31]. Within the glomerulus, both podocytes and endocapillary cells (endothelial and mesangial cells) expressed MYH9. Several different mutations in the *MYH9* gene can cause MYH9-related diseases (such as Fechtner syndrome), and some of these diseases exhibit glomerular disorder including FSGS [32]. Moreover, other *MYH9* mutations frequently found among African-Americans represent a risk factor for FSGS and hypertensive end-stage kidney disease [33]. These facts suggest that MYH9 plays a crucial role in the maintenance of structural integrity in glomerular cells. However, it remains to be seen whether renal up-regulation of MYH9 in the HFD-medaka is involved in a protective mechanism for kidney and glomerular injuries or whether the up-regulation itself contributes to the development of the glomerulopathy.

SM22α, also known as transgelin, is an actin-associated protein of calponin family predominantly expressed in the differentiated smooth muscle cell lineage [34]. In the rodent kidney, protein expression of SM22α is restricted to the smooth muscle cell of vasculature including the glomerular arterioles [35]. Under pathologic conditions inducing renal interstitial lesion induced by ischemia

Table 1
Proteins differentially expressed in the kidney between control and HFD-medaka.

		Accession No.	Identified proteins	Normalized spectral counting				
				0w	1w	2w	3w	4w
Elevated	1	olecno11_f19	Ran	4.5	3.8	4.7	5.8	8.5
	2	olebno12_a05	Receptor for activated protein kinase C	7.0	6.6	7.3	9.6	12.1
	3	olteno61_m22	Myosin, heavy chain 9, non-muscle	27.0	23.5	26.5	26.2	40.3
	4	olbrno9_c04	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	0.0	0.0	0.0	1.2	2.2
	5	olvlno65_k14	Annexin max2	3.0	2.8	2.1	3.7	7.2
	6	olbrno30_h03	Carbonic anhydrase	1.5	1.4	2.1	2.1	5.8
	7	oleano50_e23	Annexin max3	8.0	7.5	6.0	8.3	13.4
	8	olebno35_m11	Far upstream element binding protein 1	2.5	3.3	3.4	3.3	5.4
	9	olebno44_p11	Karyopherin alpha 4 (importin alpha 3)	0.0	0.0	0.0	0.4	2.7
	10	olvlno23_f21	Gsna protein	0.5	0.5	0.9	0.8	4.9
	11	olovano42_k17	Fatty acid binding protein H6-isoform	0.5	0.5	0.0	0.8	4.9
	12	olebno18_c04	Fbln1 protein	0.0	0.0	0.0	0.0	2.7
	13	olebno32_i01	Filamin-A (Filamin-1)	9.0	9.4	7.7	7.5	25.1
	14	olebno24_h17	m-Calpain	0.0	0.0	0.0	0.0	2.2
	15	olbrno15_c01	Cytochrome c oxidase subunit VIb isoform 1	1.0	0.9	2.1	2.5	3.1
	16	olovano1_j16	Radixin isoform 1	5.5	6.1	4.3	5.0	9.8
	17	olbrno39_h11	Smooth muscle cell-specific protein SM22 alpha	0.0	0.5	0.0	0.0	3.6
	18	oleano10_d23	Cytoskeletal tropomyosin	0.5	0.0	0.4	0.0	3.1
Reduced	1	olovano55_j01	Neprilysin	12.0	9.9	9.4	7.9	7.6
	2	olvlno16_h11	Enoyl Coenzyme A hydratase short chain 1 mitochondrial	5.5	4.7	4.7	4.2	2.7

and ureter obstruction, this protein is expressed *de novo* in the activated interstitial fibroblasts (myofibroblasts), as likely α-smooth muscle actin [36]. Interestingly, injured podoctyes also express SM22α in some experimental and human glomerular diseases including the HFD-induced glomerulopathy in mouse [35–37].

In the present proteomic analysis, we identified only two kinds of down-regulated protein (neprilysin and mitochondrial enoyl CoA hydratase). Neprilysin (neutral endopeptidase) is a transmembrane metallopeptidase localized within a variety of organs [38,39]. Within the kidney, neprilysin is expressed mainly at the brush border membrane of proximal tubules, and potentially within vascular smooth muscle cells, mesangial cells, endothelial cells, and fibroblasts. Neprilysin hydrolyzes physiologically active peptides including endothelin-1, substance P, and atrial natriuretic peptide [40,41]. Angiotensin-I is also metabolized by neprilysin to generate angiotensin-(1–7) [42,43], which is considered to act as a counter-regulatory peptide against angiotensin-II, and is generally believed to possess a "renoprotective" effect [44,45]. Down-regulation of neprilysin expression is likely to cause reduced production of angiotensin-(1–7), and may relatively enlarge the effects of angiotensin-II in the kidney, which is widely known to be involved in the development and aggravation of glomerular disorders including diabetes nephropathy [44–46]. It is thus possible that reduced production of angiotensin-(1–7) via the down-regulation of neprilysin partially contributes to the development of the glomerulopathy in HFD-medaka. However, since neprilysin is responsible for the metabolism of other kinds of bioactive peptides, multiple mechanisms mediated via neprilysin should be considered as potential contributors to glomerulopathy.

In conclusion, the HFD-medaka model developed a signature glomerulopathy, partially compatible to human ORG, within a relatively short term, and exhibited altered expression of several kinds of renal disease-related protein in the kidney. Thus, the HFD-medaka is a promising animal model to explore the mechanisms underlying the human ORG, and for detailed investigation of metabolic syndrome-associated glomerular disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.053.

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