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Short Communication

Ocular lesions in leptin receptor-deficient medaka (*Oryzias latipes*)

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Abstract: Ocular lesions in leptin receptor-deficient medaka were examined histopathologically at 10, 28, and 37 weeks post hatching. Leptin receptor-deficient medaka at 28 and 37 weeks old showed hyperglycemia and hypoinsulinemia. Histopathologically, vacuolation, swelling, fragmentation, and liquefaction of the lens fibers and dilatation of the retinal central veins, retinal capillaries, iridal veins and capillaries, and choroidal veins were observed in leptin receptor-deficient medaka at 28 and 37 weeks old. Thinning of the total retina, pigment epithelial layer, layer of rods and cones, outer granular layer, outer plexiform layer, inner granular layer, and inner plexiform layer was observed in leptin receptor-deficient medaka at 28 and 37 weeks compared with in control medaka. These histopathological characteristics in leptin receptor-deficient medaka are similar to characteristics in ocular lesions of rodent models for type II diabetes mellitus, making leptin receptor-deficient medaka a useful model of diabetic cataract and retinopathy. (DOI: 10.1293/tox.2017-0042; J Toxicol Pathol 2018; 31: 65–72)

Key words: cataract, hyperglycemia, leptin receptor-deficient medaka, retinopathy, vascular dilatation

The numbers and significance of diabetes mellitus cases have increased as economic development and urbanization have led to changes in lifestyles characterized by reduced physical activity and increased obesity[1]. In 2011 there were 366 million people with diabetes mellitus, and this number is expected to rise to 552 million by 2030[1]. Diabetes mellitus can cause ocular complications such as cataracts and retinopathy that frequently result in blindness[2]. An elevated prevalence of diabetes was observed among people with cataracts who were less than 70 years old, and for people with cataracts below the age of 40 years, diabetes prevalence was 15 to 25 times the prevalence in a general population[3]. Several different pathogenetic mechanisms leading to the formation of diabetic cataracts have been proposed[2]: increased osmotic stress caused by activation of the polyol pathway, nonenzymatic glycation of lens proteins, and increased oxidative stress. Diabetic retinopathy is a microangiopathy affecting all of the small retinal vessels, such as capillaries, venules, and arterioles[2]. Early clinical features of diabetic retinopathy include microaneurysms, dot and blot hemorrhages, and intraretinal microvascular anomalies[4]. As the severity of diabetic retinopathy worsens, capillary nonperfusion leads to retinal ischemia, which causes upregulation of pro-angiogenic cytokines that induce intraretinal and intravitreal neovascularization[4]. In rodents, detailed histomorphological characteristics of cataracts and retinopathy of chemical-induced diabetes mellitus and spontaneous diabetes mellitus have been described[5-10]. In fish, a previous study demonstrated histomorphological characteristics of chemical-induced diabetic retinopathy[11], but detailed histomorphological characteristics of spontaneous diabetic cataract and retinopathy have not been reported.

Leptin is a peptide hormone secreted by adipose tissues in mammals and by the liver in fish[12–16]. It has been shown to play a key role in the maintenance of energy homeostasis through the regulation of food intake, glucose metabolism, and a range of physiological functions[17, 18]. Leptin- and leptin receptor-deficient rats and mice spontaneously develop severe hyperphagia, which leads to obesity, and they display several type II diabetes mellitus-like characteristics[19]. Previously, we succeeded in producing medaka that were homozygous for leptin receptor gene mutation (leptin receptor-deficient (LRD) medaka) by targeting induced local lesions in a genome method (TILLING method)[20]. In the present study, analysis of glucose and insulin levels in the blood and histopathology of eyeballs in LRD medaka reared with ad libitum feeding at 10, 28, and 37 weeks post hatching showed retinopathy and cataracts with hyperglycemia

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Table 1. Body Weight

Age (weeks)	Control medaka (mg)	Leptin receptor-deficient medaka (mg)
10	190.25 ± 32.11	240.25 ± 34.34
28	355.86 ± 36.91	417.00 ± 44.90
37	372.00 ± 33.25	393.88 ± 32.87

Values are expressed as the mean ± SE.

Table 2. Postprandial Plasma Insulin Level

Age (weeks)	Control medaka (ng/mL)	Leptin receptor-deficient medaka (ng/mL)
28	172.75 ± 1.25	63.05 ± 7.05**
37	153.97 ± 5.52	31.17 ± 6.57**

Values are expressed as the mean ± SE. **Significantly different from the control group at $P<0.01$ (Student's *t*-test).

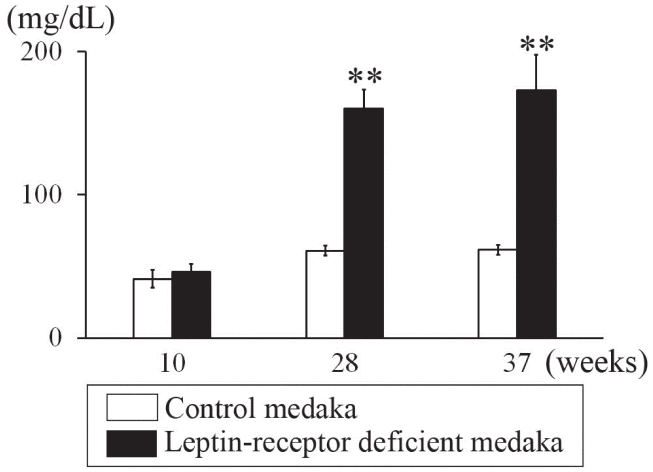

Fig. 1. Fasting plasma glucose levels. Values are expressed as the mean ± SE. **Significantly different from the control group at $P<0.01$ (Student's *t*-test).

and hypoinsulinemia at 28 and 37 weeks old. Disorders of the retina and lens were examined histopathologically in order to match histopathologic characteristics of cataracts and retinopathy in LRD medaka to those in diabetic cataract and retinopathy in rodents, and the usefulness of the LRD medaka as a model of diabetic cataract and retinopathy was evaluated.

In the present study, CAB/KYOTO-inbred substrain medaka (*Oryzias latipes*) were used as the wild type strain (control medaka). Medaka with a homozygous leptin receptor gene mutation (LRD medaka) were produced by the TILLING method as described previously[20, 21]. Fish were maintained at 25 to 26°C with a 14:10 h (light:dark) cycle in a recirculating aquaculture system equipped with carbon filtration and biofiltration. Fish were fed a standard feed for freshwater fish (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan). The present experiments were performed following the guidelines of the Animal Research Committee of Kyorin University.

At 10, 28, and 37 weeks post hatching, 18 fish (wild type, n=9; LRD, n=9) were weighed, and 36 eyeballs were examined macroscopically. After that, the fish were euthanized using 0.003% eugenol (FA100, DS Pharma Animal Health Co., Ltd., Osaka, Japan), and the blood was collected from the main artery and vein. The fasting plasma glucose levels were measured using a portable glucose meter (Nipro Care Fast Meter; Nipro, Tokyo, Japan) at 10, 28, and 37 weeks post hatching. The postprandial plasma insulin levels were measured by a method described previously[22]. Then, the fish were fixed *in toto* in Bouin's fluid overnight before postfixation with 10% neutral buffered formalin, embedded in paraffin, cut into coronal sections, and routinely stained with hematoxylin-eosin. A total of 36 eyeballs of 18 fish were examined histopathologically. The thicknesses of the total retina and retinal component layers were measured in a region 50 μm away from the optic disk with histomorphometric analysis software (Olympus Corporation, Tokyo, Japan). The number of retinal ganglion cells was counted in a high-power field at ×400 magnification with histomorphometric analysis software (Olympus Corporation).

All values are expressed as the mean ± standard error (SE). Comparisons of differences between the control medaka and the LRD medaka were analyzed using the Ekuseru-Toukei 2015 statistical software (SSRI Co., Ltd., Tokyo, Japan). The data from two groups were analyzed using the *F*-test. When variances were homogenous, the Student's *t*-test was performed. *P* values of <0.05 or <0.01 were considered indicative of statistical significance.

The body weights of the LRD medaka at 10 and 28 weeks old tended to be higher than those of the control medaka (Table 1). Fasting plasma glucose levels at 10 weeks old were the same in LRD medaka and control medaka, while fasting plasma glucose levels in the LRD medaka at 28 and 37 weeks old were significantly higher than those in the control medaka (Fig. 1). Postprandial plasma insulin levels in LRD medaka at 28 and 37 weeks old were significantly lower than those in control medaka (Table 2). There were few macro- and microscopic changes in the lens and retina of control medaka at 10, 28, and 37 weeks old and LRD medaka at 10 weeks old. Lens opacity was macroscopically observed in LRD medaka at 28 and 37 weeks old. Histopathologically, vacuolation, edema, fragmentation, and liquefaction of the lens fibers were observed in the lens cortex of LRD medaka at 28 and 37 weeks old (Fig. 2 and 3). These histopathological changes of the lens were observed in two of the six eyeballs in three LRD medaka at 28 weeks old and in all six eyeballs of three LRD medaka at 37 weeks old. The severity of these histopathological changes of the lens in LRD medaka at 28 weeks old was similar to that at 37 weeks old. In LRD medaka at 28 and 37 weeks old, dilatation was observed in the retinal central veins, retinal capillaries, iridal veins and capillaries, and choroidal veins (Fig. 4). The dilated vessels compressed the retinal parenchyma (Fig. 5). The total retina, pigment epithelial layer

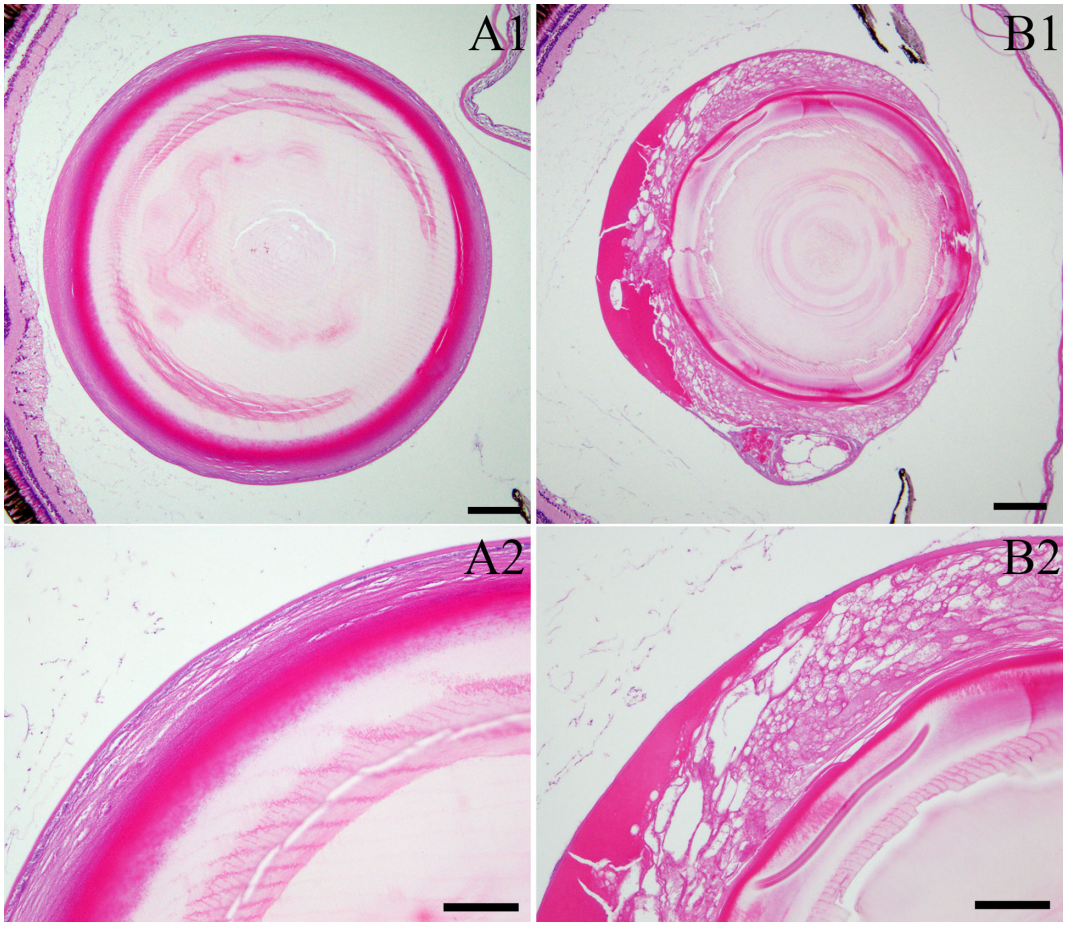

Fig. 2. Histopathological findings of the lens at 28 weeks post hatching. A: Control medaka. B: Leptin receptor-deficient medaka. Fig. 2A and 2B show magnified views of Fig. 1A and 1B. The bars in Fig. 1A and 1B are 100 µm. The bars in Fig. 2A and 2B are 50 µm.

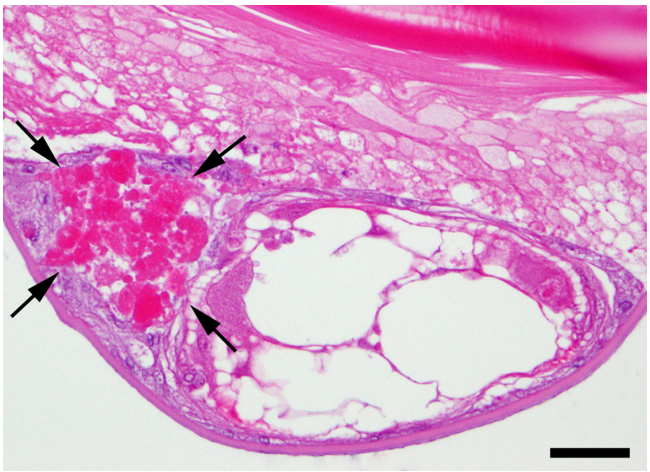

Fig. 3. High magnification view of the lens of leptin receptor-deficient medaka at 28 weeks post hatching. Arrows demonstrate fragmentation of the lens fibers. The bar is 30 µm.

(PEL), layer of rods and cones (RCL), outer granular layer (OGL), outer plexiform layer (OPL), inner granular layer (IGL), and inner plexiform layer (IPL) in LRD medaka at 28 and 37 weeks old were significantly thinner compared with those in control medaka (Fig. 6 and 7). No significant statistical differences in thickness of the nerve fiber layer (NFL) and the ganglion cell layer (GCL) and number of ganglion cells were observed between the control and LRD medaka at 10, 28 and 37 weeks old (Fig. 7 and 8). These histopathological changes of the retina were observed in all eyeballs of LRD medaka at 28 and 37 weeks old. Their severity at 28 weeks old was similar to that at 37 weeks old. No histopathological changes were observed in the cornea of the LRD medaka at 10, 28, and 37 weeks old.

In the present study, opacity of the lens, vacuolation, edema, fragmentation, and liquefaction of lens fibers were observed in the lens of LRD medaka at 28 and 37 weeks old, but these macro- and microscopic changes were not induced in control medaka throughout the study period and were not observed in LRD medaka at 10 weeks old. By comparison, these manifestations took longer to appear in rat models of type II diabetes mellitus[7, 23–25]: WBN/Kob rats, Otsuka Long-Evans Tokushima Fatty (OLETF) rats, and Spontane-

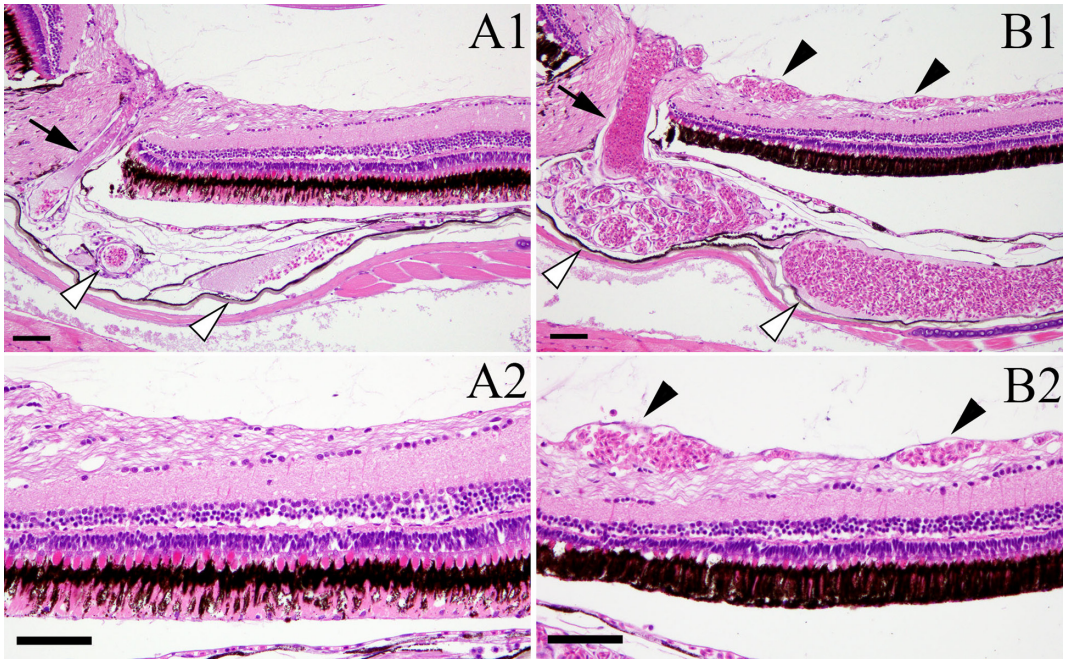

Fig. 4. Histopathological findings of the retina at 28 weeks post hatching. A: Control medaka. B: Leptin receptor-deficient medaka. Fig. 2A and 2B show magnified views of Fig. 1A and 1B. Arrows indicate retinal central veins. Arrowheads indicate retinal capillaries. White arrowheads indicate choroidal veins. The bar is 50 μm.

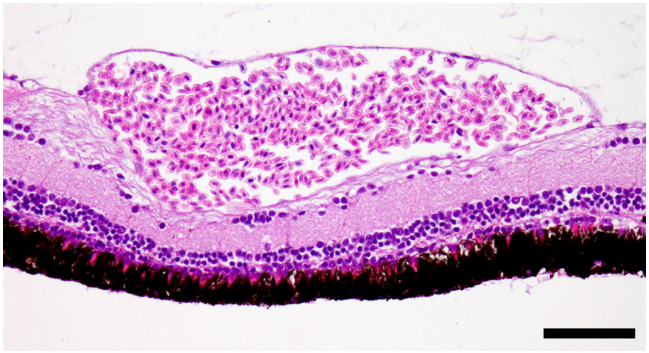

Fig. 5. High magnification view of the retina of leptin receptor-deficient medaka at 28 weeks post hatching. The dilated vessels compressed the retinal parenchyma. The bar is 50 μm.

ous Diabetic Torii (SDT) rats. Opacity of the lens began to appear at 15 months of age in WBN/Kob rats[26]. In OLETF rats, cataracts were detected at 19 months old by slit-lamp microscopy, whereas no cataracts were observed in control rats[7]. In OLETF rats, swelling and vacuolation of the cortical and supranuclear fibers were observed in the equatorial region of the lens, and the liquefaction of the cortical fiber was observed in the anterior and posterior subcapsular regions of the lens at 60 weeks old; on the other hand, there were no histopathological changes at 20 weeks old, and slight swelling of lens fibers was observed in the anterior and posterior subcapsular regions at 40 weeks old[6]. In the SDT rats, swelling, vacuolation, and disintegration of lens fibers were observed at 40 weeks of age or older[5]. The results of the present study demonstrated that in the LRD medaka, marked macro- and microscopic changes were induced within a shorter rearing period compared with rat models for type II diabetes mellitus such as WBN/Kob rats, SDT rats, and OLETF rats[5–7, 26]. Based on these characteristics, the LRD medaka is a useful model of type II diabetic cataracts.

In the present study, dilatation of the retinal central veins, retinal capillaries, iridal veins and capillaries, and choroidal veins were observed in LRD medaka at 28 and 37 weeks. Blood circulation is impaired in diabetes mellitus[27], and congestion and venous dilatation are observed in the retinas of people with diabetes mellitus[27–31]. Extensive venous dilatation was observed in the retinas of 62-week-old SDT rats[8]. The results of the present study demonstrated that in LRD medaka, extensive dilatation of retinal vessels was induced within a shorter rearing period compared with in SDT rats.

Diabetic retinopathy is classified into nonproliferative diabetic retinopathy and proliferative diabetic retinopathy[32]. Nonproliferative diabetic retinopathy is characterized by the presence of microaneurysms, intraretinal microvascular abnormalities, venous beading, loop formation, and hemorrhages[32]. Nonproliferative diabetic retinopathy develops into proliferative diabetic retinopathy, where hallmarks of neovascularization of the retina and vitreous hemorrhages are found[32]. It is considered that up to 37 weeks old, the retinopathy in LRD medaka is equivalent to the nonprolif-

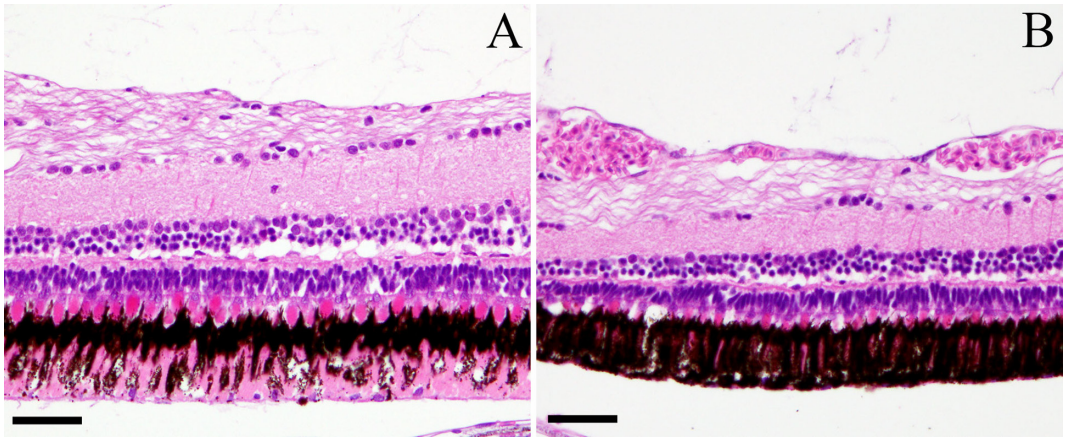

Fig. 6. Thinning of the retina of leptin receptor-deficient medaka. A: Control medaka at 28 weeks post hatching. B: Leptin receptor-deficient medaka at 28 weeks post hatching. The bar is 30 μm.

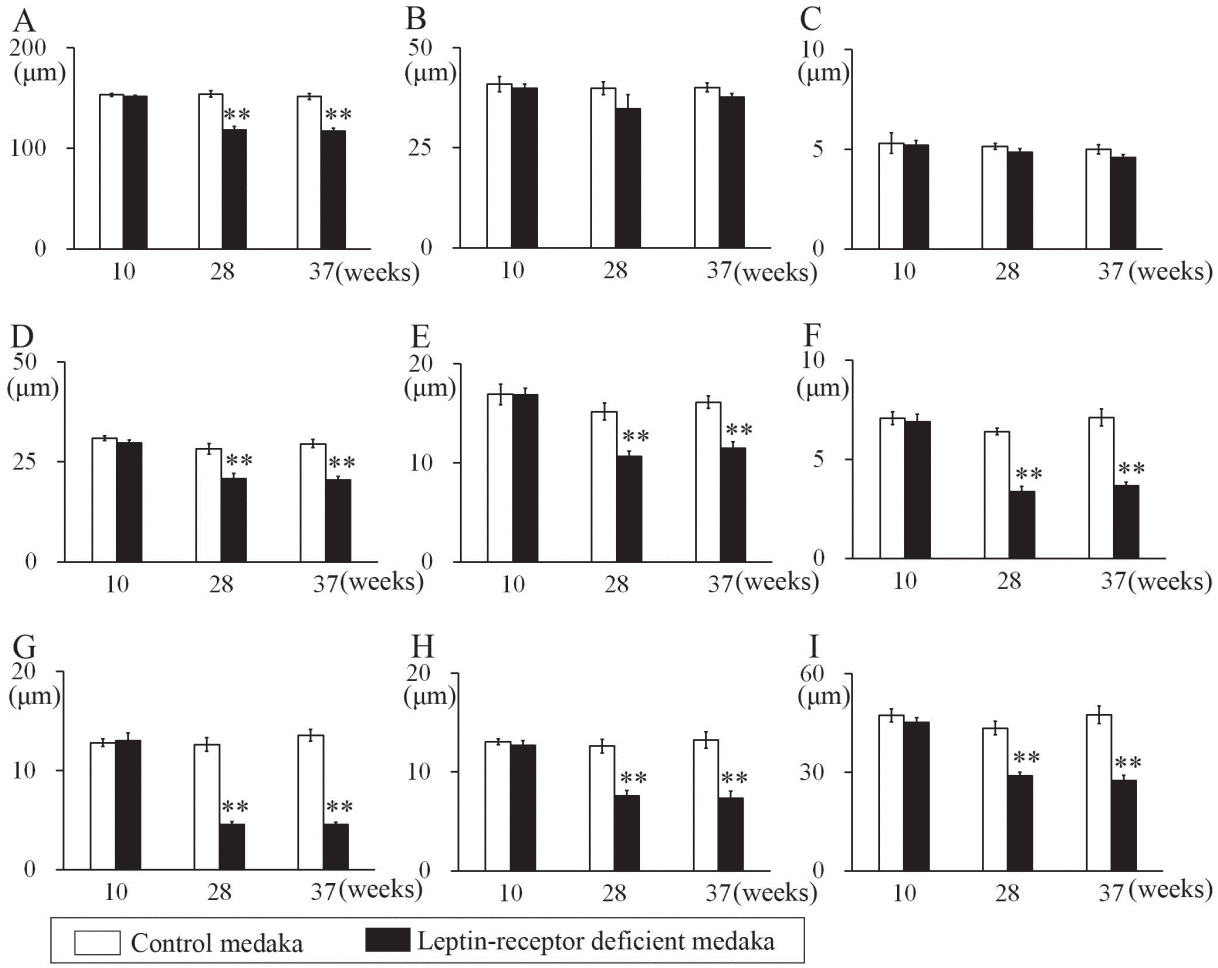

Fig. 7. Thicknesses of the total retina (A), nerve fiber layer (B), ganglion cell layer (C), inner plexiform layer (D), inner granular layer (E), outer plexiform layer (F), outer granular layer (G), layer of rods and cones (H), and pigment epithelial layer (I). **Significantly different from the control group at $P<0.01$ (Student's *t*-test).

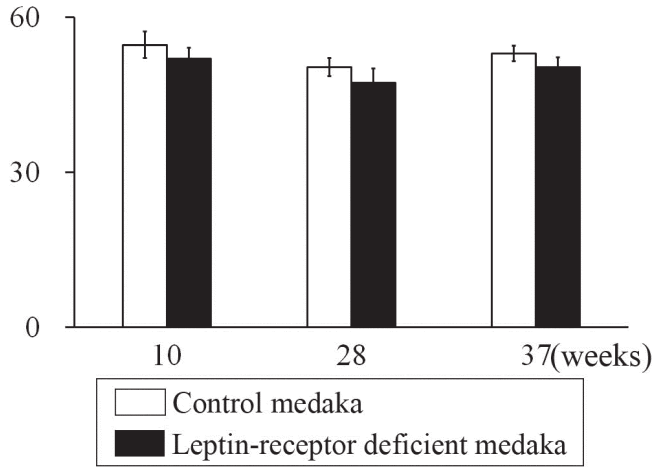

Fig. 8. Number of retinal ganglion cells in leptin receptor-deficient medaka counted in a high-power field at ×400 magnification. Values are expressed as the mean ± SE.

erative diabetic retinopathy, as no neovascularization was observed in the retinal lesions of LRD medaka in the present study.

The total retina in LRD medaka in the present study at 28 and 37 weeks old was thinner compared with that in the control medaka. In OLETF rats at 28 weeks old, the total retinal thickness was significantly less than that in control rats[9]. In the present study, the thicknesses of the PEL, RCL, OGL, and OPL in the LRD medaka at 28 and 37 weeks were significantly less than those in the control medaka. Hyperglycemia induces apoptosis of the retinal pigment epithelial cells[33, 34]. Because retinal pigment epithelial cells are crucial components of the outer blood-retinal barrier, dysfunction of retinal pigment epithelial cells, including apoptosis, is closely related to breakdown of the blood-retinal barrier, leading to the progression of diabetic retinopathy[34–37]. Additionally, retinal pigment epithelial cells interact closely with the photoreceptor outer segments[38]. Previously, apoptosis of retinal pigment epithelial cells was demonstrated to result in the progressive loss of photoreceptor neurons[38]. In the present study, hyperglycemia was suggested to primarily induce thinning of the pigment epithelial layer, which caused secondary loss of photoreceptor neurons, leading to the thinning of the RCL, OGL, and OPL. In OLETF rats at 19 months old, a decrease in the height of retinal pigment epithelial cells accompanied a decrease in photoreceptor cell nuclei[7].

The IGL consists of bipolar cells, horizontal cells, and amacrine cells[39]. Because component cells of the IGL form synapses between photoreceptor cells and interact with them, these component cells can be affected by degeneration and a decrease in the number of photoreceptor cells[39]. In the present study, thinning of the IGL and IPL may be secondarily caused by thinning of the RCL, OGL, and OPL.

In the present study, there were no significant differences in the number of retinal ganglion cells and thickness of the NFL between the LRD medaka and control medaka. However, a previous study demonstrated that the number of retinal ganglion cells and thickness of the NFL of OLETF rats decreased compared with those of the control rats[9]. These retinal histopathological changes in OLETF rats resulted from apoptosis of the retinal ganglion cells[9]. The apoptosis of retinal ganglion cells occurred via oxidative stress in streptozocin-induced diabetic rats and db/db mice[40, 41]. The reason for these histopathological differences in the retina between the LRD medaka and diabetic rodents is unclear.

In conclusion, the following changes were observed in LRD medaka at 28 and 37 weeks old: vacuolation, edema, fragmentation, and liquefaction of the lens fibers; dilatation of the retinal central veins, retinal capillaries, iridal veins and capillaries, and choroidal veins; and thinning of the total retina, PEL, RCL, OGL, OPL, IGL, and IPL. These histopathological characteristics in the lens and retina of LRD medaka were similar to those in rodent models for type II diabetes mellitus[5–9]. In the LRD medaka, the above-mentioned histopathological changes were induced within a shorter rearing period compared with in rodent models for type II diabetes mellitus[5–9, 26]. These findings demonstrate the usefulness of the LRD medaka as a model of diabetic cataract and retinopathy.

Disclosure of Potential Conflicts of Interest: We have no conflicts of interest to declare.

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