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REVIEW ARTICLE

Obese zebrafish: A small fish for a major human health condition

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Abstract

Obesity is becoming a silent worldwide epidemic, with a steady increase in both adults and children. To date, even though several drugs have been licensed for long-term obesity treatment, none of them are yet used in routine clinical practice. So far the only successful intervention has been behavioral therapy. A suitable and economic experimental model mimicking the human condition would therefore be extremely useful to evaluate preventive measures and novel treatments. Zebrafish are emerging as an important model system to study obesity and related metabolic disease. Remarkable similarities have been reported in lipid metabolism and the adipogenic pathway between zebrafish and mammals. Moreover, the zebrafish possesses a number of features—the relative inexpensiveness of animal husbandry, its optical transparency and the ability to produce a large number of offspring at low cost—that make it ideal for large-scale screening and for testing drugs and intervention. In this review, we summarize recent progress in using zebrafish as a model system to study obesity and obesity-related metabolic disorders. We describe several zebrafish models (in both larvae and adult animals) that develop obesity and non-alcoholic fatty liver disease (NAFLD) using different approaches, including gene manipulation, diet manipulation and modification of microbiota composition. For these models, we have outlined the specific aspects related to obesity and its development and we have summarized their advantages and limitations.

KEYWORDS
inflammation, metabolic diseases, NAFLD, obesity, zebrafish

1 | INTRODUCTION

Over the past decade, obesity has been increasingly recognized as silent public health problem, becoming an epidemic in many countries throughout the world.[1] Obesity can be defined as an excess of body fat. It is typically measured by body mass index (BMI) expressed as kg/m^2.[2] Using the BMI system, people are classified as overweight (BMI ≥ 25) and obese (BMI ≥ 30). Obesity is a consequence of a prolonged imbalance between introduced calories and energy expenditure. The resultant energy surplus is stored as lipid droplets throughout the body, in both adipose and non-adipose tissues. Several data indicate that a "junk dietary pattern," involving individual genetics, lifestyle and "junk" food consumption, and excess caloric intake contribute to the increased occurrence of obesity and

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obesity-related diseases.[3,4] The current recommendations for the treatment of obese people include lifestyle modification, increasing physical activity and reduction of caloric intake. Pharmacotherapy can be considered if these interventions are ineffective. Food and Drug Administration (FDA)-approved drugs for the treatment of obesity are currently 5: bupropion-naltrexone, liraglutide, lorcaserin, orlistat, and phentermine-topiramate. However, the consistent number of adverse effects still discourages their routine clinical use.[5]

The pathogenesis of obesity is not completely clear. It involves processes far more complex than the passive accumulation of fat excess throughout the body.[6,7] Adipose tissue (AT) responds rapidly and dynamically to an excess of nutrients through adipocyte hypertrophy and hyperplasia. Systemic physiology reacts to AT changes with modification of endocrine and metabolic functions. In particular, AT remodeling has been investigated to clarify the relationship among pro-inflammatory adipocytokines, cytokines and obesity-associated metabolic disorders.[8] Macrophages play a main role as the immune cells responsible for AT inflammation. Obesity induces a phenotype switch in macrophage polarization and increases secretion of pro-inflammatory molecules such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1).[8-10] In addition, the interaction between adipocytes and macrophages results in marked down-regulation of anti-inflammatory adipocytokines. The process leads to development of obesity-related complications in multiple organs,[11] that is a progressive increase in the risk of metabolic disorders like insulin resistance, type 2 diabetes mellitus (T2DM), cardiovascular diseases, stroke, and several types of cancer.[11,12]

Several metabolic studies have revealed that the regional distribution of fat within the body is more relevant than the total amount of body fat in predicting the development of obesity-related comorbidities.[13,14] In particular, the amount of visceral adipose tissue (VAT) and the ectopic deposition of fat are critically correlated with elevated risk of development of cardiovascular and metabolic disorders.[14] These obesity-related disorders are commonly known as metabolic syndrome and are present in the majority of obesity cases.[15] Plasmatic free fatty acids (FFAs) released by increased AT in obese patients form an important link between obesity and metabolic syndrome.[16] Increased flux of FFAs to the liver and muscle promotes lipotoxicity, alters insulin action and leads to non-alcoholic fatty liver disease (NAFLD), a spectrum of liver tissue changes ranging from steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma.[4]

It is therefore of the greatest importance to develop a suitable and economic experimental model that enables investigations of the pathogenic mechanisms of obesity and evaluation of drug interventions. Over the past few decades, several animal models have provided a fundamental contribution to our understanding of the genetic and nutritional factors that regulate the expansion of AT in the obesity condition. Among these models, *Danio rerio* (zebrafish) is emerging as important vertebrate system in which to study obesity and related metabolic disease, to discover and investigate the origin and progression of these conditions, and to test new potential drugs aimed at modulating the pathways involved. As a model for biomedical research, including considerations of economy of animal husbandry and rapidity of biological events, the zebrafish is a powerful instrument that reflects several anatomic, genetic and functional features of mammals.[17] Moreover, the similarity between the cellular anatomy of zebrafish adipocytes and mammalian AT and the presence of all key organs required in lipid metabolism makes it an ideal tool to study adipogenesis, obesity and metabolic disease.[18,19] Furthermore, in zebrafish, microbiota can be easily modulated. As in mammals, microbiota have been shown to have an important role in obesity and obesity-related diseases. For all these reasons, several models of obesity and metabolic disease have been developed in zebrafish, which have led to the identification of genes of interest and to the evaluation of drugs with potential to regulating lipid metabolism and AT accumulation (Figure 1).

2 | IN VIVO APPROACH TO STUDYING OBESITY IN ZEBRAFISH LARVAE

2.1 | Lipid staining

To better understand the alterations in the physiology of an organism caused by obesity, the zebrafish model can be used to trace the origin of adipogenesis, from egg fertilization to larval state. In mammals, there are two main classes of AT: brown adipose tissue (BAT), which dissipates energy and generates heat, and white adipose tissue (WAT), designed for energy storage and regulation of energy balance. Unfortunately, the mechanisms involved in their development and regulation are still unclear[20] due in part to difficulties associated with imaging AT in mammalian model systems, especially during early life stages.

Zebrafish larvae, developing outside the mother's body and being optically transparent, are suitable tools for the microscopic and biochemical study of lipid transport and for screening defects in lipid uptake, transport, and disposition. Indeed, lipid metabolism, obesity, cardiovascular damage, and metabolic diseases have been extensively studied in this model.[17,21] Zebrafish possess all specialized cell types involved in lipid absorption and processing (eg intestinal enterocytes, fat-storing adipocytes, hepatocytes in the liver, and acinar cells of the pancreas).[22,23] Adipocytes can be visualized in developing zebrafish larvae with various dyes, such as the sudanophilic dye Oil Red O (ORO), Sudan, Nile Red or Lipid Green.[24] To assess fatty acid mobilization and transport, the lipophilic BODIPY® fluorophore is used: when injected into the zebrafish yolk, it rapidly diffuses within 3 hours into the circulatory system.[25]

Zebrafish larvae start to eat at 5-6 days post-fertilization (dpf); during this period, they absorb essential fat-soluble vitamins, triacylglycerol (TAG) and cholesterol provided by a maternally derived yolk sac.[26] At the 5 dpf larvae stage, lipids are present in many tissues, including a minor accumulation in hepatocytes. The first signs of adipogenesis become visible at 8 dpf in the visceral cavity close to the pancreas. Adipocytes also appear in 12 dpf larvae in the pancreas.[26]

Using Nile Red staining, adipocytes are clearly visible at 15 dpf in the visceral region (intra-abdominal and surrounding internal

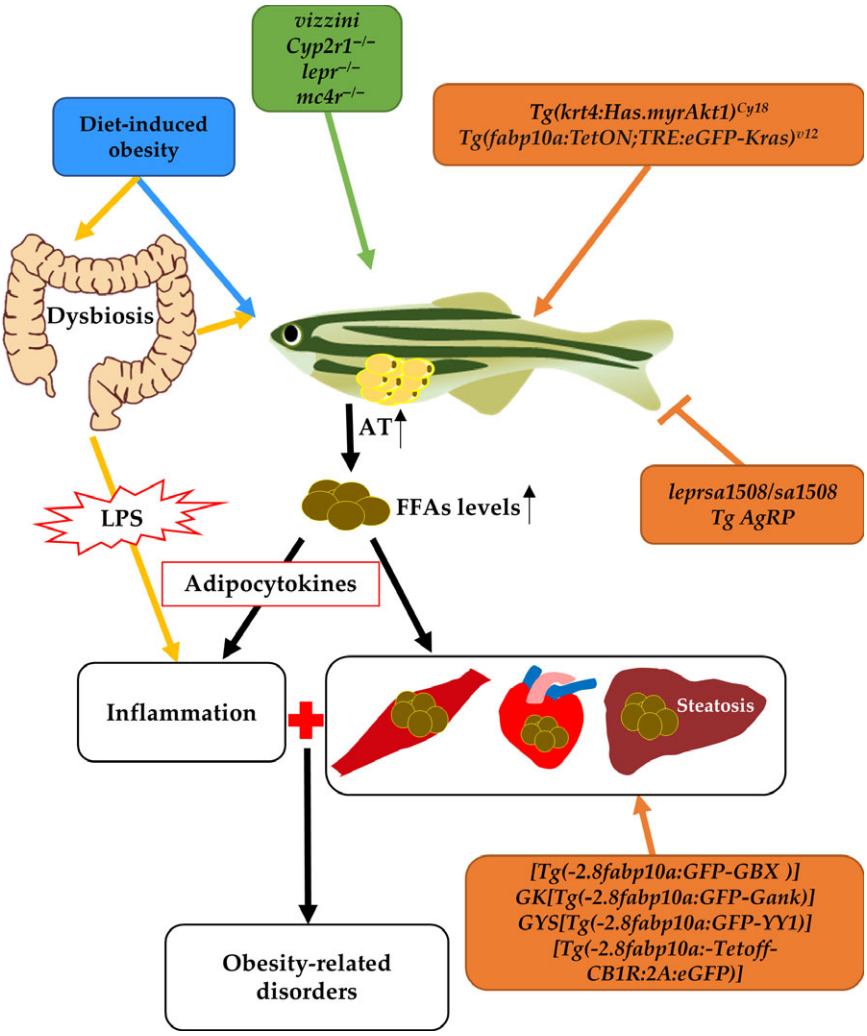

FIGURE 1 Zebrafish models of obesity and obesity-related disease. Several different approaches, including diet-induced obesity (blue), and mutant (green) and transgenic (red) models, are used in zebrafish to study the pathways involved in obesity development and progression. Proadipogenic and antiadipogenic pathways are described and used in order to characterize this condition at the molecular level. Increased adipose tissue (AT) causes a rise in free fatty acids (FFAs) that determines ectopic fat deposition and inflammation. Both phenomena collaborate in inducing obesity-related diseases. Microbiota dysbiosis is involved by promoting obesity development and inflammation. Transgenic models of lipid accumulation in the liver, which resemble the human condition, were also developed in zebrafish but they failed to induce obesity

organs),[20] a localization associated in humans with more adverse risk factors for developing T2DM.[27,28] The number of adipocytes is correlated with size of the larvae rather than age, suggesting body-length dependent lipid storage in adipocytes. Whereas at 17 dpf, all larvae have WAT in the pancreatic and visceral area, at 20-22 dpf they develop, in a size-dependent manner, subcutaneous (SL > 8.2 mm) and cranial (SL > 9.4 mm) adipocytes.[29]

Lipid staining allows monitoring of dietary lipid absorption following a high-fat liquid feeding protocol in zebrafish larvae: after incubation for 1 hour in a 4% solution of fat (containing heavy whipping cream) the anterior intestine and inter-segmental vessels of 6 dpf larvae exhibited strong staining with ORO. The increase in the level of ORO staining correlated with whole-larval TAG.

2.2 | In situ hybridization and mRNA markers of obesity

In response to a single high-fat meal, microsomal triglyceride transfer protein (*mtp*) mRNA levels increase in the proximal intestine and liver, but the protein level remains unchanged.[30] Marza and collaborators[31] reported that MTP protein with 54% identity with human MTP is present in zebrafish. Using in situ hybridization and RT-PCR, they confirmed developmental regulation and tissue specificity of mtp expression during early embryogenesis and in anterior intestine and liver from 48 hpf onward. MTP is involved in lipoprotein assembly into nascent ApoB. The complex ApoB-MTP-lipoproteins is able to prevent the degradation of lipids by proteasomes and to increase plasma lipid levels. As expected, ApoB levels increased in response to feeding.[32,33] All the evidence mentioned above suggests that WAT distribution in zebrafish larvae and its regulation have a conserved molecular basis with humans.[34] The process of adipogenesis has been well studied and most master regulators have been identified: teleost adipocytes express genes associated with lipid metabolism such as fatty acid binding protein 11a (*FABP11A*), peroxisome proliferator-activated receptor gamma (*PPARG*), and CCAAT/enhancer binding protein alpha (*CEBPA*),[23,30,34-36] and adipocyte endocrine function (*LEPTIN* and *ADIPONECTIN*).[30,36-38] In particular, the FABP11A protein plays an important role in maintenance of glucose and lipid homeostasis as well as angiogenesis.[39] PPARs are regulators of lipid metabolism, with an important role in energy release through lipid breakdown.[36] PPARG is involved in lipid storage and adipogenesis, and also in differential regulation of insulin resistance and glycemic control.[40] *Pparγ* mRNA is expressed early in zebrafish development (5-10 hpf). The transcription factor C/EBPα seems to

control *PPARG* expression. These factors drive adipocyte differentiation. In zebrafish, *cebpα* mRNA is clearly expressed in both visceral and pancreatic WAT, and, interestingly, in unfertilized eggs.[30]

A recent study reported, for the first time, that the transcription factor SOX6 acts as an activator of adipogenesis upstream of *PPARG* and mesoderm specific transcript (MEST) by direct binding to their promoters. The basic functions of SOX6 in adipogenesis are conserved between humans and other vertebrates, as shown in vivo in sox6 homozygous null-mutant larvae of zebrafish. These findings are an important step in investigating a hypothetical direct role of the fetal origins of human obesity and its postnatal development.[41]

2.3 | Gene manipulation to follow gene expression in vivo

One of the major advantages of zebrafish is the simplicity and efficiency of manipulation of gene expression. Mutant and transgenic fish, in which gene expression has been deregulated, allow in vivo studies at a molecular level of the pathway involved in diseases. Moreover, transgenic zebrafish larvae can express fluorescent proteins in specific cell types, which are then easily detected in the transparent larvae, enabling monitoring of obesity development and progression. Furthermore, zebrafish embryos and larvae up to 5 dpf are not subject to the same regulatory requirements as adult mammals, increasing the value of this high-throughput instrument.

Using transgenes (Table 1), eg, zebrafish *Tg(hPPARγ-eGFP)*[42] incorporating green fluorescent protein (GFP) under regulatory control of the gene of interest, it is possible to monitor live tissues and to investigate specific drug actions. An interesting study conducted in this model has tested the obesogenic potential of chemicals that we are exposed to on a daily basis in our environment, evaluating lipid accumulation and its correlation to their capacity to activate *PPARG* in zebrafish larvae.[43]

Recently, several studies reported a key role of macrophages in adipose tissue inflammation. Different molecules involved are identified, but the underlying mechanism still remains unclear. The zebrafish macrophage reporter line *Tg(lyz:Ds Red)* has been used to study macrophage infiltration into adipose tissue in zebrafish.[44] *Lysozyme C* (*LysC)* expression has been reported to specifically mark the macrophage compartment on the larvae yolk. Studies based on fluorescent reporters would help scientists to elucidate, using in vivo monitoring, inflammatory activation mechanisms and processes that induce transformation from healthy to pathological adipose tissue.

Obesity is the single most significant risk factor for the development of steatosis and zebrafish larvae develop steatosis. The incidence and degree of steatosis were more severe in fish fed with a high fat diet containing cholesterol (HFC). Hepatic lipid accumulation in larvae increases the reliability of the HFC model for screening of potential anti-steatosis drugs.[45]

Another advantage of zebrafish larvae is the creation of transient knockdown of gene expression through the use of morpholinos (MOs),[46] which allows rapid and effective study of gene function. However, this is limited to the early stages of development. For instance, larvae injected with an MO-ApoC2 (Apolipoprotein C2) exhibit an unabsorbed yolk phenotype. As result of reverse genetics in zebrafish, apoC2, necessary for lipoprotein assembly in humans, has been found to be essential during zebrafish larval development.[47] Fat mobilization in response to starvation and refeeding have also been studied in zebrafish larvae. It was observed that, when fish were starved for 4 days, neutral lipid depots were reduced in all locations, until all fat materials were dissolved at 7 days of starvation. Refeeding for 4 days was sufficient to re-establish neutral lipid depots in the same locations as before starvation.[24] These observations are consistent with data in humans.

The zebrafish transgenic bioluminescence reporter *Tg(pck1:Luc2)* is used for the identification of metabolically active drugs in the gluconeogenesis process in response to the 'feeding to fasting' transition. The cytosolic phosphoenolpyruvate-carboxykinase (*PCK1*) promoter is induced when dietary carbohydrates are low. Its protein catalyzes a regulatory step in gluconeogenesis in the liver and kidney. *PCK1* transcription is under insulin control during feeding conditions and under glucagon, glucocorticoid and adrenaline control during fasting conditions.[48] These models can be used for further investigation, and will be of particular importance in this age of eating disorders and unhealthy diets.

3 | DIET-INDUCED OBESITY MODEL IN ADULT ZEBRAFISH

Animal models that closely mimic the human obese condition and that allow the characterization of the metabolic pathways involved in obesity development are of great interest. Most of them are diet-induced obesity models. These models recapitulate the gradual weight gain that occurs in human populations as consequence of a positive energy balance over years. Oka et al[49] created the first model of diet-induced obesity in adult zebrafish in 2010. Zebrafish overfed with high quantity of *Artemia nauplii* (a common live feed used in zebrafish facilities) quickly developed a significant increase in BMI. At the end of treatment, they exhibited hypertriglyceridemia and hepatic steatosis.[49] In this study, the authors did not explore the long-term effect of overfeeding. In our lab, we set up a model based on long-term exposure to a hypercaloric diet. We observed that zebrafish acquired not only NAFLD, but also liver steatosis, which was followed by development of fibrosis. Overfed fertile female zebrafish developed less steatosis and were protected from the development of fibrosis compared to overfed old females and all males.[50] In humans, different studies have demonstrated that non-alcoholic hepatic steatosis is less common in women than in men and that the prevalence of NAFLD is lower in women of reproductive age.[50] This is consistent with accumulating evidence for the role of inflammation in the transition from NAFLD to NASH.[51] Reproductive status is associated with a generally lower level of inflammation while estrogen levels are high.[50] This observation suggests the possibility of using zebrafish in the evaluation of fibrosis (and other disease) development during different reproductive stages, with the

TABLE 1 Transgene zebrafish models: (1) three different constructs of fluorescent reporters (GFP- DsRed- Luciferase); (2) liver specific expression constructs obtained by insertion of fapb10a promoter sequence; (3) model of ectopic gene expression to induce obese phenotype

Zebrafish reporter lines (1)	Description	Gene of interest	References
Tg(hPPARγ-eGFP)	Green fluorescent protein (GFP) under regulatory control of peroxisome proliferator-activated receptor gamma (PPARγ)	PPARγ is a regulator of adipocyte differentiation. Additionally, it is implicated in the pathology of obesity, diabetes, atherosclerosis and cancer	42
Tg(lyz:Ds Red)	Red reporter DsRED2 under control of lysozyme C (LysC) gene expression	*Lysozyme C* (*LysC*) expression occurs in the macrophage compartment, on the zebrafish larvae yolk. Marker of the macrophage lineage	44
Tg(pck1:Luc2)	Luciferase expression under the cytosolic phosphoenolpyruvate-carboxykinase (PCK1) promoter	*PCK1* is a control gene for gluconeogenesis regulation. The expression of this gene can be regulated by insulin, glucocorticoids, glucagon, cAMP, and diet	48
Zebrafish liver-specific lines (2)	Transgene	Gene of interest	References
Tg(−2.8fabp10a:gfp-gank) *Tg(−2.8fabp10a:HBV.HBx-GFP)* *Tg(−2.8fabp10a:EGFP-yy1b)*	The liver fatty acid binding protein (L-FABP) allows liver specific expression of green fluorescent protein (GFP)	Gankyrin (*gank*) Hepatitis B virus X protein Ubiquitous transcription factor Yin Yang 1 (YY1) Modification of their expression is able to induce liver steatosis in zebrafish	82-85
Tg(fabp10a:TETA-cnr1,EGFP)	Conditional expression by use of a liver-specific (fabp10a) Tet^{off} transgenic system	Cannabinoid receptor 1 (CB1R): responsible for food intake and weight gain and regulates several pathological features associated with obesity in mammals	86
Tg(fabp10a:TetON;TRE:eGFP-kras)^{v12}	Conditional expression by use of a liver-specific (fabp10a) Tet^{on} transgenic system	Upregulation of Ras signaling is able to determine the accumulation of lipid droplets in zebrafish hepatocytes and increase in the amount of TG	88
Zebrafish lipid metabolism-related line (3)	Transgene	Gene of interest	References
Tg(krt4:Has.myrAkt1)^{Cy18}	AKT1 under the keratine4 promoter	The ectopic expression of AKT1 in adipocytes is responsible for the obese phenotype	87

additional advantage that ovarian senescence in zebrafish is spontaneous.[50,52]

An additional constant feature in both models was the extremely high penetrance of development of obesity and fatty liver. This is in contrast with the rodent models, in which only a fraction of treated animals developed these conditions.[53,54] Interestingly, zebrafish were fed in a meal-feeding scheme that is very similar to human food habits. A similar approach has also been proposed in rodents but the use of diets that vary in nutrient composition, energy density, consistency, and flavor gave rise to inconsistent data, with substantial differences between experimental animals. Only when food was continuously made available, on a day and night basis, did obesity consistently develop.[55]

Microarray analysis of liver tissue from zebrafish models of diet-induced obesity reveals a gene expression pattern that resembles human NAFLD.[56] Comparisons between DNA microarray analysis of zebrafish and mammalian VAT revealed that genes involved in blood coagulation, platelet activation and lipid metabolism are significantly deregulated in obese zebrafish and mammals (mouse, rat and human). Moreover, diet-induced obesity in zebrafish responds to caloric restriction and to treatment with natural compounds (green tea, Campari tomato, resveratrol) by reducing BMI and modifying the expression of genes related to obesity.[57-59]

Obesity is also positively associated with dietary factors such as increased fat intake; in humans a high-fat diet (HFD; >30% of energy in fat) can easily induce obesity. This is also true in rats and mice where a positive relation has been observed between the amount of fat in the diet and body weight and fat gain.[60,61] The role of dietary lipids has also been assessed in zebrafish. Adult zebrafish on a high cholesterol diet become obese and display an increased deposition of adipocyte in the abdominal region.[62] In order to evaluate the effect of dietary fat on body fat accumulation, Meguro et al[63] compared the effect of four different diets (respectively enriched in starch, gluten, corn oil, and lard) on zebrafish body fat volume. No differences were observed in feed efficiency among groups, but zebrafish treated with a diet enriched in fat (corn oil and lard) displayed greater body fat volumes, consistent with previous experiments in rodents. Indeed, in rodents the quantity of dietary fat, but not the type of fat or the total energy intake, was shown to be responsible of body fat accumulation.[61] Differences in fat deposition and a predisposition to developing obesity-related metabolic disease are described in human patients.[64] Some patients exhibit so-

called "metabolically healthy obesity," which is characterized by the absence of metabolic abnormalities and comparatively less VAT and low infiltration of macrophages into adipose tissue, as well as smaller adipocyte cell size.[65] This phenotype contrasts with the 'metabolically unhealthy obese' phenotype which exhibits a deleterious AT distribution, with more visceral fat, larger adipocytes and evidence of inflammatory processes.[66] A comparison between zebrafish fed a high-fat diet (HFD) or a normal-fat diet (NFD) showed that both dietary regimens can induce a significant increase in BMI. An MRI analysis of fat distribution revealed larger visceral and smaller subcutaneous adipocytes in HFD overfed fish compared to NFD obese animals. HFD overfed zebrafish also showed significantly elevated blood glucose, triglyceride and cholesterol levels and a prominent accumulation of lipids in liver and muscles.[67] These data suggest that, depending on the type of diet and food regimen, zebrafish could resemble both the "healthy" and "unhealthy" obesity phenotypes and provide an interesting model for studies of the regulatory mechanisms involved in these pathways, as well as the metabolic states.[67]

Recently, a model of T2DM in zebrafish has been established.[68] To develop a diet-induced obesity model with hyperglycemia, fish were fed with an increased quantity of a commercial food. In terms of calories, each overfed animal was provided with 408 calories/day vs. the 150 calories/day used by Oka et al.[49] This regimen is able to very quickly induce an increase in BMI, adipose tissue volume and plasma triglyceride, as well as an impaired glucose tolerance, increased insulin production and an increased β-cell mass, consistent with an insulin resistance model of T2DM. Remarkably, this model of T2DM is responsive to antidiabetic drugs (metformin and glimepiride) and has transcriptomic pathways similar to human disease.[69]

All the cited models of diet-induced obesity in zebrafish raise the possibility using this animal to study the development of obesity and obesity-related disease in a system that resembles the human/mammalian pathology. Unfortunately, a standardized diet for common application in zebrafish has not yet been formulated.[70] As reported in Table 2, several diet regimens differing in calories, fat content and composition have been used so far. Diets that vary in their nutrient composition give rise to differences in energy intake, body composition and AT deposition. In addition to complications arising from variable diets, husbandry conditions can also affect energy expenditure and, as a consequence, caloric balance.[55,70]

4 | ZEBRAFISH MUTANTS AS A MODEL OF OBESITY

Mutant fish are currently used in order to study obesity-related disease. Zebrafish mutants that carry mutations in genes involved in AT storage and in mobilization are good tools for further characterization of cellular and molecular mechanisms involved in fat accumulation, in order to identify molecular targets and approaches needed to treat or prevent obesity (Figure 2).

The *vizzini* mutant carries a null mutation in the *GH1* gene that encodes for an endocrine factor produced by the anterior pituitary gland, which serves to synchronize growth and to regulate lipid metabolism.[71] As a consequence, animals display an increase in AT and the inability to properly mobilize stored fats. The same

TABLE 2 Different protocols of diet-induced obesity (DIO) in zebrafish and the resultant phenotype

Model of DIO zebrafish	Feeding	Observation period	Phenotype
Oka et al[49]	60 mg/cyst/d *Artemia Nauplii*	8 wk	Obesity, increased plasma triglyceride, alteration in genes involved in lipid metabolism and coagulation
Meguro et al[63]	LF (low fat diet) Otohime B2 + starch or gluten HF (high fat diet): Otohime B2 + lard or corn oil	6 wk	Increased body fat volume and body fat volume ratio in HF-fed animals
Forn-Cunì et al[92]	*Artemia Nauplii* +fish chow (4 meal/d)	8 mo	Obesity, steatosis, liver gene expression similar to human NAFLD
Turola et al[50]	60 mg/cyst/d *Artemia Nauplii*	24 weeks	Obesity, liver steatosis and fibrosis
David et al[62]	HCD (high cholesterol diet): Pelleted fish food + 6% cholesterol	6-8 wk	Increased BMI, obesity, lipid droplets (LDs) in intestinal microvilli
Zang et al[68]	120 mg/d Otohime B2 (commercial fish food)	8 wk	Insulin resistant T2DM, with glucose intolerance and insulin overproduction
Landgraf et al[67]	NFD (normal fat diet): 60 mg/cists/d *Artemia Nauplii* HFD (high fat diet): 5 mg/cists/d *Artemia Nauplii* + 30 mg egg yolk powder	8 wk	Increased body weight and AT mass in both diets Hyperglycemia and ectopic lipid accumulation in HFD fed fish

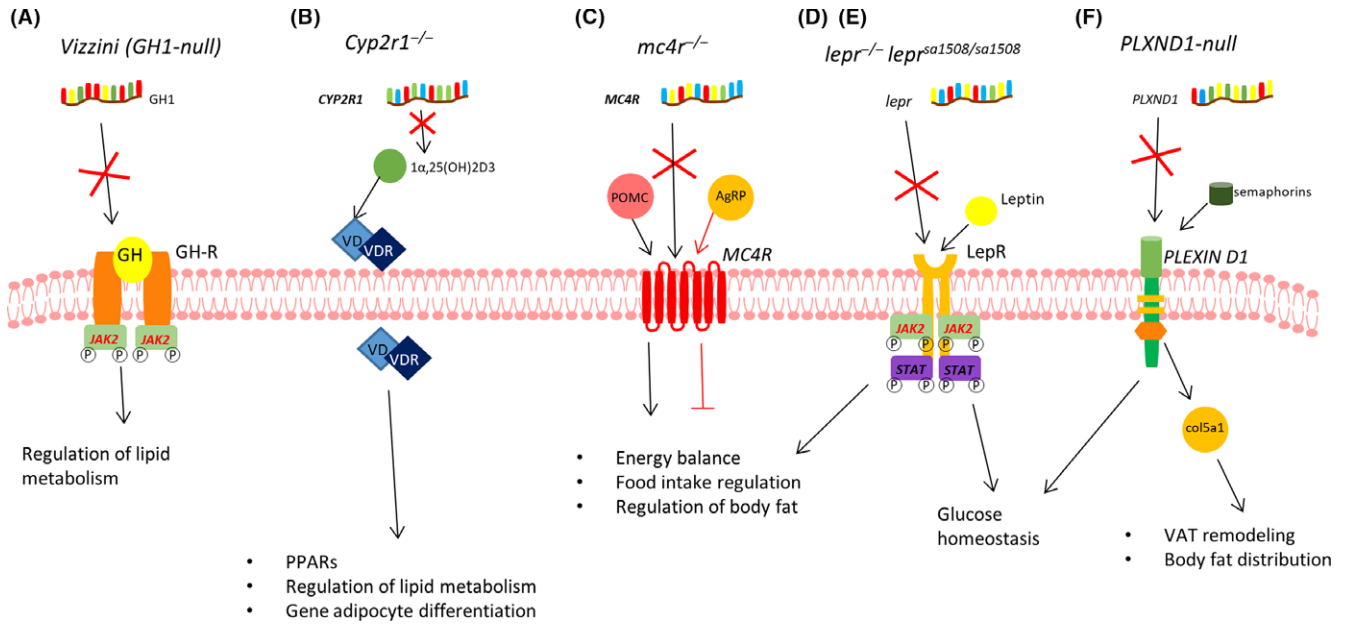

FIGURE 2 Zebrafish mutant models of obesity and fat distribution. Several mutant models in zebrafish are available in order to study the pathways involved in obese phenotypes. The signaling of different models is shown in the figure: (A) *vizzini*, (B) *cyp2r1*$^{-/-}$, (C) *mc4r*$^{-/-}$, (D) *lepr*$^{-/-}$, (E) *leprsa1508/sa1508*, (F) *PLXND1-null*. The main effects of these pathways are connected to regulation of lipid metabolism, control of energy balance, distribution of body fat and/or sensitivity to insulin

characteristics were observed in GH-deficient and GH-insensitive mouse. Another zebrafish mutant used in studying obesity is *cyp2r1*$^{-/-}$, which has a premature stop codon in the CYP2R1 gene.[72] Like the *vizzini* mutants, *cyp2r1*$^{-/-}$ zebrafish displayed excessive VAT accumulation, mainly due to an increase in number (but not size) of adipocytes and significantly increased total lipids. Supplementation with 1,25(OH)2D3 reverses the defect caused by the mutation, suggesting that vitamin D3 is an important regulator of lipid metabolism.[72] Accordingly, mice defective in the vitamin D/vitamin D receptor system display a lean phenotype, and resistance to HFD-induced obesity.[73] In addition, the *lepr* and *mc4r* genes are obesity-related. Mutations of these genes in zebrafish, achieved via the CRISPR/Cas9 technique, have shown that the adult *lepr*$^{-/-}$ and *mc4r*$^{-/-}$ individuals, but not post-juvenile fishes, displayed characteristics of obesity phenotypes like higher weight and body fat percentages, as well as significantly impaired glucose tolerance.[74] MC4R and leptin protein are involved in feeding and metabolism, and seem to be conserved between teleost fish, including zebrafish, and mammals.[75,76] In particular, MC4R mediates the melanocortin system, playing an important role in the evolution of the diverse array of reproductive, growth, and feeding strategies in teleost fish.[77] Experimental blockade of MC4R by overexpression of agouti-related protein (AgRP), or mutations in pro-opiomelanocortin (POMC), an endogenous agonist of AgRP, increased body weight, body fat and adult length in zebrafish, as well as and levels of triglyceride.[76,78,79] The pathway driven by the leptin hormone is also known to be implicated in energy homeostasis and obesity regulation, and to be active in different circuits in the brain involved in regulation of food intake, energy balance and glucose homeostasis.

Leptin deficiency in mammals (due to mutation in both leptin or leptin receptor genes) is a well-known cause of obesity, diabetes and infertility, while leptin replacement in leptin-deficient individuals ameliorates obesity.[38] In zebrafish, a mutant for the leptin receptor (*lepr*$^{sa1508/sa1508}$) has been isolated through screening for mutations after *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis.[38] In contrast to data collected in rodents, adult zebrafish with a mutated leptin receptor do not exhibit increased adiposity. Indeed, no differences in body composition and amount of fat were observed between wild type and *lepr*$^{sa1508/sa1508}$ zebrafish, irrespective of whether they were overfed or fed on a standard diet. On the other hand, mutant zebrafish displayed increased levels of insulin mRNA, alterations in glucose homeostasis, increased β-cell mass, and altered wound healing, but not insulin resistance.[38] Leptin receptor-deficiency (knockout) was also established in medaka.[80] As in the *lepr*$^{-/-}$ and *mc4r*$^{-/-}$ zebrafish models,[74] in the *lepr*$^{-/-}$ medaka model, the post-juvenile and adult stages responded differently to food intake. In the post-juvenile stage, the mutant exhibited a higher growth rate, indicating that increased intake of food was effectively utilized for growth from post-hatching until the adult stage, and not for lipogenesis. In contrast, at the adult stage, the increased food intake led to excessive fat deposits only around the abdominal viscera, with no alteration of TAG in liver, skeletal muscle or in the bloom. These data suggest that zebrafish could also be used to study the regulation of food intake by neuroendocrine modulators and the effects on fat accumulation leading to obesity.

Another protein in zebrafish not directly correlated with obesity, but with body fat distribution, is Plexin D1. Inhibition of Plexin D1 (PLXND1) led to different effects in zebrafish and mouse models of obesity.[81] Indeed, PLXND1 knockout mice died at birth, while *PLXND1-null* zebrafish were vital, allowing the study of gene ablation in adult animals. In zebrafish, PLXND1 regulates body fat distribution by determining the growth characteristics of VAT. The absence of PLXND1 results in a preferential expansion of subcutaneous adipose tissue (SAT) in response to HFD, and thus leads to further exacerbation of altered body fat distribution, decreasing the VAT:SAT ratio. Thus PLXND1 deficiency protects zebrafish from HFD-induced insulin resistance and glucose intolerance, in accordance with association data from humans.[81]

5 | TRANSGENIC ZEBRAFISH IN THE STUDY OF OBESITY-RELATED DISEASE

In order to study obesity-related disease, transgenic zebrafish displaying altered accumulation of AT and liver steatosis were developed. The goal of these models was to mimic a specific pathology (ie, liver steatosis) to characterize the pathway involved in the disease and to study its downstream effects. Three transgenic models for studying liver steatosis and NASH were developed in zebrafish by Her et al. In these models gankyrin, HBx and YY1 were expressed, associated with GFP, under the liver-specific L-FABP 2.8 promoter.[82-85] All these three models in adult zebrafish displayed hepatic steatosis due to increased transcriptional activity of lipogenic genes and subsequent increased hepatic de novo FFA synthesis and lipid accumulation in hepatic cells. This initiated apoptosis, lipid oxidation and reactive oxygen species (ROS), leading to further development of liver damage. Interestingly, miRNA, known to be associated with NASH, was also found to be deregulated in transgenic zebrafish expressing gankyrin.[82] A similar phenotype, characterized by hepatic steatosis and alterations in the lysogenic gene *SPREBP-1c*, the lipogenic enzyme involved in fatty acid synthesis, transporters, and lipid storage genes, was also observed in the conditional transgenic zebrafish [*Tg(-2.8fabp10a:-Tetoff-CB1R:2A:eGFP)*], without treatment with doxycycline.[86] Despite the increased de novo synthesis of FFAs and the high rate of lipid accumulation in the liver, no obesity has been observed in these models. Gankyrin and HBx transgenic fish display emaciation and a declining growth rate, probably as a consequence of liver disease.[82,83]

In contrast, a severe obese phenotype has been observed in adult *Tg(krt4:Has.myrAkt1)*^{Cy18} zebrafish, which express the human *AKT1* gene under the keratin 4 promoter.[87] The ectopic expression of AKT1 in adipocytes is responsible for the obese phenotype. AKT1 is known to act upstream of PPARγ and CEBPα in modulating adipogenesis. Transgenic animals display hyperplastic growth of adipocytes, increased total triglyceride content, glucose intolerance, fat tissue accumulation in dorsal muscles, gills, and tail, neutrophil infiltration and osteoporosis caused by adipocyte infiltration. Moreover, *Tg(krt4:Has.myrAkt1)*^{Cy18} zebrafish also displayed a reduced activity (observed by swimming behavior assay) and a short life span.[87] As reported by Yao et al[88] transgenic zebrafish that conditionally activate Kras (*Tg(fabp10a:TetON;TRE:eGFP-kras)*^{v12}) developed intensive accumulation of lipid droplets in hepatocytes and increased amounts of TG. Taken together, these data suggest not only that the ectopic transgenic modification of Ras and/or Akt expression is able to cause NAFLD in the liver, but also that these pathways could be investigated as possible therapeutic targets for blocking the progress of liver damage.

The availability of these stable transgenic models provides a promising tool for studying the molecular pathways involved in liver disease and a simple and reliable method for assessing drug treatment efficacy.

6 | ROLE OF MICROBIOTA IN OBESITY CONTROL

In addition to various other causal agents, alterations in intestinal microbiota have recently been linked to the induction and progression of liver damage.[89] Gut flora alterations also include overproduction and release into the circulation of bacterial endotoxins (lipopolysaccharides, LPS). Evidence suggests that the variation (increase in the number and/or alteration in the type of bacteria) in the intestinal microbiota can increase gut permeability and LPS plasma levels.[90] The liver transcriptomic response to LPS has been evaluated in overfed zebrafish.[91] The host inflammatory response to gut microbiota is emerging as an important factor in the development of NASH.[92-96] Obese zebrafish showed a small increase in the expression of pro-inflammatory genes, but they are not able to completely activate the entire inflammatory pathway involved, probably due to the basal chronic inflammation process induced by metabolic factors such as obesity.[92] Differences in and modifications to microbiota through probiotic or prebiotic drugs are able to predict the responsiveness to weight-loss diets and pharmacological treatment in obese individuals.[97,98] In order to understand the correlation between intestinal microbiota and obesity and obesity-related conditions, the use of germ-free animals is very useful. In 2008, a method for generating gnotobiotic wild-type and/or genetically manipulated zebrafish for the study of symbiotic/commensal host-bacterial relationships in the vertebrate digestive tract was developed.[99] The relative ease of obtaining suitable experimental conditions, compared to mouse model experiments, together with the possibility of monitoring biological processes in vivo and the similarity to mammalian microbiota composition, made germ-free zebrafish an attractive tool for studying gut microbiota. Dietary lipid content has been shown to influence the gut microbiome in adult zebrafish, suggesting that it is strictly related to adiposity and obesity. The exposure of adult zebrafish to different amounts of lipid (high [HFD], medium [MFD], low [LFD]) shifted the philotype composition of the gut microbiome by reducing community diversity in the HFD group, and affected the transcription of genes involved in appetite control and in cholesterol metabolism suggesting an important role for gut microbiota in

obesity.[100] Notably, supplementation of the diet with the probiotic *Lactobacillus rhamnosus* decreases total body cholesterol in zebrafish fed with HFD and MFD,[100] suggesting new possibilities in the treatment of obesity and high-fat diet-related metabolic disorders through manipulation of gut microbiota.[100,101] These data suggest that the gnotobiotic zebrafish model could be a useful tool for exploiting the functions of intestinal microbiota in the control of obesity and its related diseases and to test how modulation of microbiota could affect the pathology.

7 | CONCLUSION

Zebrafish is emerging as a relevant system in the study of obesity and obesity-related disorders. Several mutant and transgenic lines that display alterations in AT deposition and mobilization, and in the regulatory brain circuits involved in its homeostasis, have been set up in larvae and adult zebrafish. These models are very useful to characterize the mechanisms and pathways involved in the diseases and to test new pharmacological and therapeutic approaches. However, they fail to mimic the initial stage of obesity development; indeed, human obesity is mostly a consequence of a positive energy balance, primarily due to excessive food intake. The animal models that best reflect the human pathology are dietary-induced models. Interestingly, zebrafish respond well to diet modification. Exposure to hypercaloric and high fat diets quickly induces obesity and obesity-related disease and activates metabolic pathways very similar to their human counterparts. Unfortunately, the lack of standardized feeding protocols and housing conditions leads to variation in the results obtained by diet manipulation and is still a great limitation of these models.

CONFLICT OF INTEREST

None.

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