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Title
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Permalink
https://escholarship.org/uc/item/9p45g29n

Journal
Translational Research, 163(2)

ISSN
1931-5244

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Publication Date
2014-02-01

DOI
10.1016/j.trsl.2013.09.004

Peer reviewed

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Transl Res. Author manuscript; available in PMC 2015 February 01.

Published in final edited form as:
Transl Res. 2014 February ; 163(2): 99–108. doi:10.1016/j.trsl.2013.09.004.

Zebrafish models of dyslipidemia: Relevance to atherosclerosis and angiogenesis

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Abstract

Lipid and lipoprotein metabolism in zebrafish and in humans are remarkably similar. Zebrafish express all major nuclear receptors, lipid transporters, apolipoproteins and enzymes involved in lipoprotein metabolism. Unlike mice, zebrafish express *cetp* and the Cetp activity is detected in zebrafish plasma. Feeding zebrafish a high cholesterol diet, without any genetic intervention, results in significant hypercholesterolemia and robust lipoprotein oxidation, making zebrafish an attractive animal model to study mechanisms relevant to early development of human atherosclerosis. These studies are facilitated by the optical transparency of zebrafish larvae and the availability of transgenic zebrafish expressing fluorescent proteins in endothelial cells and macrophages. Thus, vascular processes can be monitored in live animals. In this review article we discuss recent advances in using dyslipidemic zebrafish in atherosclerosis-related studies. We also summarize recent work connecting lipid metabolism with regulation of angiogenesis, the work that considerably benefited from using the zebrafish model. These studies uncovered the role of *aibp*, *abca1*, *abcg1*, *mtp*, *apoB* and *apoC2* in regulation of angiogenesis in zebrafish and paved the way for future studies in mammals, which may suggest new therapeutic approaches to modulation of excessive or diminished angiogenesis contributing to the pathogenesis of human disease.

INTRODUCTION

Consumption of a high cholesterol, high fat diet and genetic mutations contribute to dyslipidemia, which, in turn, is a major risk factor for atherosclerosis and cardiovascular disease. Disorders of lipid metabolism also result in dysregulation of important physiologic processes and play a role in the pathogenesis of adipose tissue inflammation and insulin resistance, diabetes, steatohepatitis, renal disorders, and neurodegenerative diseases. Rodent animal models were instrumental in uncovering causes of dyslipidemia and elucidating mechanisms of diseases linked to altered lipid metabolism. Work from our laboratory contributed to the development of a zebrafish model of hypercholesterolemia and vascular lesion formation, relevant to early stages of human atherogenesis. Zebrafish were also used to model other aspects of lipid metabolism and to elucidate mechanisms of disorders associated with dyslipidemia. In this article, we will focus on recent work in which zebrafish were used to study the connection between lipid metabolism and vascular pathology, specifically, disorganized angiogenesis and vascular lipid accumulation and lesion formation.

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The advantages of a zebrafish model include the optical transparency of zebrafish embryos and larvae and the availability of effective tools of genetic manipulation. Together, they enable *in vivo* monitoring of vascular processes in live transgenic zebrafish expressing fluorescent proteins in specific vascular cell types and/or fed a diet supplemented with fluorescent lipid tracers. For example, Table I lists several studies in which transgenic zebrafish with EGFP or DsRed expressed in endothelial cells (EC), myeloid cells or macrophages were used, often in conjunction with fluorescent lipids and lipoproteins. Other advantages include economic colony maintenance, large numbers of progeny obtained from a single mating and that zebrafish larvae readily absorb small molecules from water.

More importantly, notable similarities in human and zebrafish lipid metabolism, propensity to hyperlipidemia and lipoprotein oxidation make zebrafish a particularly attractive animal model for studying mechanisms of diseases triggered by dyslipidemia. The studies related to dyslipidemia and vascular pathology, highlighted in this article, contribute to the growing trend of using zebrafish models for defining disease pathways and for discovering new therapies in a broad range of human disorders [1].

LIPOPROTEIN AND LIPID METABOLISM IN FISH

Zebrafish express all the major classes of apolipoproteins, apoA, apoB, apoC and apoE, which share high homology with human apolipoproteins [2-4]. Antibodies against human apoB-100 and apoA-I recognize proteins of a similar molecular mass in zebrafish plasma and were used in a plate-based immunoassay to capture zebrafish lipoproteins [3, 5]. During zebrafish embryonic development, yolk syncytial layer actively synthesizes apoE as well as other apolipoproteins, and produces VLDL from yolk lipids, which subsequently enters the circulation and transfers nutrient lipids to the whole body [2]. As in mammals, zebrafish microsomal triglyceride transfer protein (Mtp) functions in the VLDL assembly in the yolk during early development [6]. The presence of VLDL, LDL and HDL lipoprotein fractions was documented in rainbow trout and zebrafish plasma, using density gradient ultracentrifugation and agarose gel electrophoresis [3, 7]. HDL dominates the lipoprotein profile in zebrafish fed a normal diet. However, feeding a high cholesterol diet (HCD) increases the proportion of VLDL and LDL fractions in zebrafish plasma and results in a lipoprotein profile closely resembling that of human plasma [3].

Fish favor lipids rather than carbohydrates as the source of energy and are hyperlipidemic and hypercholesterolemic compared to mammals [7]. Apolipoproteins comprise as much as 36% of total proteins in fish plasma compared to only 10% in humans. Compared to humans, there is more triglycerides (TG) but less cholesterol esters (CE) in fish LDL [7]. Zebrafish express *cetp*, homologous to the human cholesterol ester transfer protein gene *CETP*, and the Cetp activity in zebrafish plasma increases with hypercholesterolemia [8, 9]. In fact, the presence of Cetp is an important advantage of the zebrafish model compared to mice, which do not express CETP, and may be one of the reasons why feeding zebrafish a HCD results in notable hypercholesterolemia, as discussed below. CETP is considered an important therapeutic target for human dyslipidemia, although recent clinical trials have questioned the importance of CETP inhibitor therapies. In addition, the activities of lipoprotein lipase, hepatic lipase and lecitin:cholesterol acyltransferase (LCAT) have been documented in several fish species [7]. A homolog of human proprotein convertase subtilisin/kexin type 9 (PCSK9) is involved in cholesterol homeostasis in zebrafish [10]. The SLC16A6 transporter of β-hydroxybutyrate plays an important role in fasting metabolism in zebrafish [11], although the function of its homolog in humans remains unexplored.

Recent studies started to explore zebrafish homologs of mammalian nuclear receptors involved in lipid metabolism. As in mammals, zebrafish PPARα can be activated by a

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putative agonist clofibrate to induce the expression of fatty acid binding protein genes *fabp2*, *fabp3* and *fabp6* [12]. Zebrafish LXR can be activated by the agonist of human LXR T0901317 to trigger typical anti-inflammatory responses [13]. Both cholesterol transporters Abca1 and Abcg1 have been found in zebrafish [14-16] and the gene expression level of zebrafish *abca1* is regulated by LXR activity as well [16].

There are also similarities between zebrafish and humans in the intestinal cholesterol absorption. Zebrafish express Npc1l1, a homolog of human Niemann-Pick C1-like protein 1 (NPC1L1), which is involved in intestinal absorption and transfer of cholesterol and is a target of the cholesterol lowering drug ezetimibe. Dietary fatty acids promote intestinal cholesterol absorption by rapid re-localization of Npc1l1 to the intestinal brush border [17]. The ezetimibe-binding domain of Npc1l1, with the phenylalanine and methionine residues critical for high-affinity binding of ezetimibe, is conserved in zebrafish. Administration of ezetimibe reduces intestinal cholesterol levels in zebrafish by 25-75% [18]. In our studies, ezetimibe was very effective in reducing total cholesterol levels in HCD-fed zebrafish larvae. In contrast, simvastatin, which inhibits HMG CoA reductase (HMGCR), was less effective in reducing cholesterol levels in zebrafish [19]. The Ffr protein encoded by the *fat-free* gene and involved in Golgi vesicular trafficking has been identified as an important factor in intestinal lipid absorption by zebrafish [20]. Ffr also regulates phospholipase D activity and contributes to zebrafish glucose metabolism as well [21]. Although zebrafish intestinal physiology is under-explored, recent advances in understanding the role of intestinal regulation in mammalian lipid metabolism [22, 23] make this topic particularly important.

MODELING EARLY PROCESSES OF ATHEROSCLEROSIS IN HYPERCHOLESTEROLEMIC ZEBRAFISH

Atherosclerosis is a chronic inflammatory disease driven in large part by hypercholesterolemia and often manifested in myocardial infarction or stroke. Hypercholesterolemia, induced in zebrafish by feeding them a HCD, initiates vascular processes characteristic of early development of human atherosclerosis. Thus, as early as 5 days after starting HCD feeding, we observed accumulation in zebrafish vasculature of a fluorescent cholesterol ester or a fluorescent analog of free cholesterol, which were added to the HCD. The majority of lipid deposits were accumulated in macrophages and possibly in other myeloid cells, similar to lipid-laden foam cells in human atherosclerotic lesions [3, 24-26] (Fig. 1A). These lipid deposits and macrophages were localized outside the lumen and beneath the endothelial cell (EC) layer, but as yet we are unable to conclude with certainty that the macrophages were inside the vessel wall intima because zebrafish with fluorescent protein expression in vascular smooth muscle cells are thus far unavailable. Yoon et al. reported that prolonged HCD feeding of adult zebrafish resulted in elevated IL-1β expression in spleen and liver, but that the vascular lipid accumulation in HCD-fed larvae was not accompanied with increases in inflammatory cytokines (IL-1β or TNFα) in total body homogenates [27]. These results indicate that a short HCD feeding may not induce systemic inflammation in larvae but they do not exclude local, vascular inflammatory responses to vascular lipid accumulation.

It is believed that some vascular locations are prone to atherosclerosis due to higher levels of LDL penetration. Xie et al. injected a red fluorescent DiI-LDL into *flk1:EGFP* embryos and recorded the hemodynamics of the injected LDL using a laser scanning confocal microscope [28]. The authors found that the concentration of LDL was increased at the vascular wall compared to the center of the lumen, suggesting a polarized LDL distribution. Furthermore, the LDL concentration at the artery bifurcations, where lipid deposits tend to accumulate, was significantly elevated compared to the linear areas of the vasculature. Consistent with

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our findings, the injected DiI-LDL accumulated in the caudal vein 3 days after injection [28].

In addition to monitoring LDL distribution in the lumen, vascular lipid accumulation and recruitment of macrophages, and macrophage foam cell formation, hypercholesterolemic zebrafish were used to detect elevated vascular activity of phospholipase A_2 (PLA_2) [3], using the same fluorogenic PLA_2 substrate that was used in studies of the digestive system in zebrafish [29]. The HCD feeding also resulted in the thickening of the EC layer in zebrafish vasculature and increased vascular permeability as detected with intravascular injections of a high molecular mass fluorescent dextran [3].

MONITORING OXIDIZED LIPIDS IN HYPERCHOLESTEROLEMIC ZEBRAFISH

It is widely recognized that oxidized LDL (OxLDL) is atherogenic and that LDL oxidation drives the initial formation of atherosclerotic lesions in both humans and experimental animals [30, 31]. Hypercholesterolemic zebrafish manifests extremely high levels of OxLDL, as is evident from high mobility LDL bands in native agarose gel electrophoresis and from binding of the anti-oxidized phospholipid antibody E06 [3]. We characterized oxidized lipids in the homogenates of HCD-fed zebrafish larvae using LC-MS/MS [5]. The levels of non-oxygenated CEs (cholesteryl arachidonate, linoleate and oleate) were 4–13-fold higher in HCD-fed larvae than in larvae fed control diet, which is likely due to increases in circulating lipoproteins and/or retention of lipid in tissues [5]. Notably, oxidized CEs were increased as much as 10–70-fold, suggesting that hypercholesteromia in larvae leads to profound lipoprotein oxidation [5]. Importantly, the OxCE molecular species present in HCD-fed zebrafish have the same retention time and mass as those in lipid extracts from human and mouse atherosclerotic lesions as well as in plasma of patients with cardiovascular disease [5, 32, 33]. Many OxCE components are proinflammatory and proatherogenic [34, 35]. We further analyzed non-oxidized and oxidized phosphatidylcholines (PC) in hypercholesterolemic zebrafish larvae. The levels of many lysoPC and OxPC, but not non-oxidized PC, increased 5-25 folds [5]. The OxPC characterized include 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-(5, 6-epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC), and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC). These OxPC have been shown to induce proinflammatory responses in vascular cells and are present in human and mouse atherosclerotic lesions [30, 36-40].

Although biochemical characterization and immunological assays of oxidized lipids in zebrafish provide important insights into lipid oxidation processes, direct visualization of oxidized lipids in live transparent zebrafish larvae would offer obvious advantages. Therefore, we developed a method, which exploited a diet supplemented with fluorescent lipids and fluorescently tagged oxidation-specific antibodies, to examine accumulation of lipid deposits and oxidation epitopes in vivo. The antibody we used, IK17, was cloned in the Witztum laboratory from a patient with advanced cardiovascular disease [41]. IK17 has been shown to bind malondialdehyde (MDA)-modified LDL and other MDA-modified proteins and to stain mouse and human atherosclerotic lesions [41-43]. IK17-reactive epitopes are present in thin cap fibroatheroma and in advanced or ruptured human lesions [44]. To test whether vascular lipid deposits in HCD-fed zebrafish contain MDA-modified proteins, we employed two methods. First, we injected recombinant, fluorescently labeled IK17 into HCD-fed zebrafish larvae. A single chain Fv fragment (scFv) was engineered from the heavy and light chains of the IK17 Fab fragment and the resultant scFv IK17 antibody was produced in *E. coli*, followed by its labeling with Alexa Fluor 488 [24]. The labeled scFv IK17 bound to the MDA epitopes in vitro and to selected areas of vascular lipid deposits in

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HCD-fed zebrafish as well [24]. The partial colocalization of lipid deposits and IK17 signals in larvae suggests that MDA epitopes accumulate only in particular areas of vascular lesions, which agrees with the selective IK17 staining of mouse and human atherosclerotic lesions.

As a second approach to visualize oxidized lipids in live zebrafish larvae, we engineered a transgenic zebrafish with conditional expression of IK17 [24]. Using this transgenic zebrafish allows for a simple and efficient manipulation of antibody expression, repeatedly and at various time points. It also offers the opportunity to study therapeutic effects of diets, antioxidants, and of oxidation-specific antibodies themselves, without tedious steps of purification and injection of recombinant antibody. The *hsp70:IK17-EGFP* zebrafish that we generated, transiently express IK17-EGFP only after heat shock (1 hour at 37°C) [24]. IK17-EGFP is secreted into the circulation and binds to the vascular lipid deposits (Fig. 1A). By using *hsp70:IK17-EGFP* zebrafish, we found that HCD feeding-induced accumulation of oxidized lipids in the vasculature can be reversed by switching back to the control diet. Further, we treated *hsp70:IK17-EGFP* zebrafish with the antioxidant probucol, which is a potent lipophilic antioxidant reducing atherosclerotic burden in hyperlipidemic rabbits but not cholesterol levels in plasma [45, 46]. We found that probucol treated zebrafish had less vascular lipid accumulation. Remarkably, probucol also reduced IK17-positive vascular lesions, resulting in many lipid deposits devoid of IK17-reactive epitopes [24]. Our studies are congruent with other antioxidant studies that were performed in zebrafish. For example, since zebrafish cannot synthesize vitamin C, feeding zebrafish a vitamin C-deficient diet led to robust oxidative stress as demonstrated by increased MDA levels, as measured by a thiobarbituric acid reactive substances assay [47].

ANGIOGENESIS IN ZEBRAFISH

Recent studies suggest that disorders of lipoprotein metabolism are important not only for the formation of vascular lesions, leading to atherosclerosis, but also for the embryonic vasculature development and vascular remodeling (Table II). Blood vessels can be established in several ways, among which angiogenesis is the physiological process of new blood vessel formation from pre-existing arteries or veins. Angiogenesis also plays a critical role in pathogenesis of many diseases. For example, dysregulated angiogenesis contributes to obesity and atherosclerosis [48, 49]. Angiogenesis in atherosclerotic plaques leads to their growth and renders the plaques vulnerable to rupture [49]. Tumor growth is supported by extensive angiogenesis to satisfy the ever growing demand for nutrients in cancer cells [50]. Insufficient angiogenesis in the myocardium after myocardial infarction is the cause of ischemia. The angiogenesis therapy has been applied clinically to promote growth of collateral blood vessels in ischemic heart and promote revascularization in hypertrophic heart [51]. In addition, the angiogenesis treatment is applied to patients after high risk percutaneous coronary intervention or coronary artery bypass surgery [51].

Zebrafish microangiography and imaging of transgenic zebrafish that express EGFP driven by EC-specific promoters have been extensively used to dissect the molecular mechanisms that govern angiogenesis [52-54]. The cardiovascular system in zebrafish does differ from that of mammals. However, the development of zebrafish vasculature and the anatomy formation proceed in a very similar fashion to that of mammals [55]. The molecular pathways that are responsible for embryonic angiogenesis are conserved in zebrafish, and most of the key players in angiogenesis have been identified in zebrafish, including Vegf and Vegf receptors, Semaphorin-3 and receptors of the PlexinD family, Tie1-2, Notch family members, as well as transcription factors Cloche, Scl, Fli1 and Runx [56-66]. The tissue expression patterns and functions of these angiogenic genes are homologous to those of mammals. Because angiogenesis also occurs pathophysiologically, extensive studies have

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been carried out to perturb these signaling pathways to either promote or inhibit angiogenesis.

Sprouting angiogenesis is driven by two types of EC: Tip cells extend philopodial protrusions and migrate toward angiogenic cues, of which VEGF is a major growth factor, and stalk cells proliferate and form the lumen of a new blood vessel. The VEGF signaling occurs via its major receptor VEGFR2 in tip cells but it is inhibited in stalk cells [67]. In contrast to VEGFR2, VEGFR1 is a decoy receptor for VEGF and is documented to negatively regulate tip cells proliferation, migration and branching via the Notch pathway [68]. In addition, expression of the transcription factor HIX-1 dictates stalk cell specification in sprouting angiogenesis [69].

LIPOPROTEIN METABOLISM AND REGULATION OF ANGIOGENESIS IN ZEBRAFISH

Our recent study suggests that maintaining cholesterol balance in EC is essential for proper angiogenesis [14] (Fig. 1B). We have also identified apoA-I binding protein (AIBP) as a regulator of cholesterol efflux from EC, which restricts angiogenesis in vitro and in embryonic zebrafish. Specifically, AIBP, which is a secreted protein, accelerates cholesterol efflux from human umbilical vein endothelial cells (HUVEC) to HDL, the effect that depends on the presence of the cholesterol transporter ATP binding cassette (ABC) G1 [14].The mechanism by which AIBP accelerates cholesterol efflux to HDL is only partially understood. AIBP does not bind cholesterol but increases HDL binding to HUVEC, even at a 1:125 molar ratio [14]. This may suggest an enzymatic function of AIBP. Indeed, recent papers report structural data and results of test-tube assays that imply a function of AIBP as an NAD(P)HX epimerase or an ADP-ribosyl transferase [70, 71], although the in vivo enzymatic function of AIBP is yet to be demonstrated.

Cholesterol efflux regulates angiogenesis via modulation of lipid rafts and VEGFR2 signaling in EC. When applied together but not individually, AIBP and HDL significantly reduced the abundance of lipid rafts in HUVEC, resulting in reduced VEGFR2 localization to lipid rafts and VEGFR2 dimerization, endocytosis and downstream signaling, culminating in the inhibition of VEGF-stimulated angiogenesis. Furthermore, AIBP/HDL inhibited VEGF-stimulated mouse aortic ring neovascularization ex vivo [14]. If cholesterol removal is the sole mechanism that results in inhibition of angiogenesis, one would expect that increasing concentration of HDL would be sufficient and achieve the same results as with AIBP/HDL. However, experimental evidence suggests that HDL alone promotes rather than inhibits HUVEC angiogenesis in vitro. One explanation is that bioactive lipids, such as sphingosine-1-phosphate (S1P), carried by HDL, promote angiogenesis in vitro [72, 73]. In contrast, another study showed that HDL in the concentration range of 40-80 mg/dL impaired angiogenesis. The authors demonstrated that HDL promoted human endothelial progenitor cell (EPC) senescence and retarded in vitro angiogenesis by activation of Rho-associated kinase (ROCK) and inhibition of PI3K/Akt and p38/MAPK pathways. ROCK inhibitors or the HMGCR inhibitor statins rescued high HDL-induced EPC senescence and facilitated angiogenesis of EPCs [74]. However, the effect of statins was assigned to the inhibition of Rho, but not to the reduction of intracellular cholesterol levels.

Cholesterol is removed from the cells via cholesterol transporters ABCA1 and ABCG1 to apoA-I and HDL, respectively. During zebrafish embryonic development, the yolk supplies nutrition to the whole body and basically functions as a liver, where all the lipoprotein synthesis occurs. In zebrafish, knockdown of *aibp* or *abca1* and *abcg1* leads to increased levels of free cholesterol. In agreement with the in vitro studies, examination of angiogenic

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pathways clearly demonstrated activation of the VEGF signaling in *aibp2* and *abca1/g1* morphants [14].

During zebrafish angiogenesis of segmental arteries, the tip cells proliferate and migrate dorsally following a permissive pathway and respond to guidance cues, directing the movement of the sprouts [67]. In contrast, stalk cells remain attached to the dorsal aorta and do not migrate further [52, 75]. We found a higher membrane lipid order, which roughly corresponds to lipid rafts, in tip cells compared to stalk cells. Aibp deficiency increased, and HDL administration decreased the membrane lipid order. Accordingly, reduced Aibp expression in zebrafish resulted in excessive sprouting/branching angiogenesis, whereas Aibp forced expression inhibited angiogenesis [14]. In agreement with the role of cholesterol efflux in regulation of angiogenesis, cholesterol transporter Abca1/Abcg1-deficient embryos displayed dysregulated, extensive angiogenesis as well. Although in the HUVEC experiment ABCG1 was the major effector of the AIBP effect on cholesterol efflux and angiogenesis, Abca1 was the dominant cholesterol transporter responsible for aberrant angiogenesis in zebrafish [14]. One explanation could be that HDL is formed initially from lipid poor apoA-I, which accepts cholesterol via ABCA1. Subsequently, HDL accepts cholesterol via ABCG1. Therefore loss of ABCA1, compared to the loss of ABCG1, would inhibit cholesterol efflux to the most extent because there would be no acceptor for ABCG1 either. On the other hand, ABCG1 but not ABCA1 has been suggested to remove cholesterol from lipid rafts [76].

In contrast to the apoA-I-containing HDL, the apoB-containing LDL and VLDL deliver cholesterol and other lipids to the cells. Thus, following the logic of the cholesterol efflux/ angiogenesis study discussed above [14], one would expect that increased LDL levels will facilitate angiogenesis. Interestingly, Avraham-Davidi et al. found that, on the contrary, apoB lipoproteins negatively regulated angiogenesis in zebrafish embryos [4]. The authors identified a mutation, stalactite (*stl*), in the gene encoding Mtp, which is involved in VLDL assembly in the yolk. The *stl* mutants displayed a dysregulated angiogenesis phenotype similar to that of *aibp* and *abca1/abcg1* deficient zebrafish. Hyperlipidemia achieved by knockdown of *apoC2* or by injection of LDL or by transplanting HEK293 cells expressing apoB into the subintestinal vascular bed, abolished the excessive sprouting phenotype. Further, the authors found that addition of long, median or short chain fatty acids had no role in impeding angiogenesis. They concluded that the apoB protein but not lipid components of LDL is responsible for the inhibition of angiogenesis in hyperlipidemic zebrafish. The effect of apoB on angiogenesis was due to regulation of Vegfr1 expression, which is a decoy receptor for Vegf. In agreement, VEGFR1 was downregulated in hyperlipidemic mice [4]. The remaining intriguing question of this study is how does apoB regulate Vegfr1 expression and whether LDL internalization is required for this mechanism. An earlier report showed that *mtp* knockdown did not result in any dramatic vascular abnormality [6]. This discrepancy could be explained by an incomplete knockout of *mtp* in the knockdown experiment and that a small amount of Mtp may be sufficient to support normal angiogenesis, albeit the *mtp* knockdown embryos did display the loss of Oil Red O staining compared to controls [6].

Similarly to mouse studies, zebrafish treated with the HMGCR inhibitor aplexone showed attenuated sprouting in the caudal vein plexus, and this effect depended on the geranylgeranylation of RhoA. As expected, incubation with the downstream product of HMGCR mevalonate reversed the angiogenic phenotype caused by HMGCR inhibition. Surprisingly, aplexone had no effect on arterial angiogenesis [77]. Our preliminary data show that atorvastatin has an effect similar to that of aplexone on angiogenesis. However, simvastatin, even at a low dose, was lethal to zebrafish upon longer exposures [19]. This

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could be due the skeletal muscle toxicity of statins in zebrafish [78]. Therefore, zebrafish phenotypes observed with prolonged statin treatments should be interpreted with caution.

CONCLUSIONS

The studies discussed in this article suggest that using zebrafish is emerging as a powerful and unique animal model to dissect new molecular mechanisms and pathways that contribute to dyslipidemia and associated disorders. These disorders include, traditionally, atherosclerosis and, rather unexpectedly, angiogenesis. The optical transparency of zebrafish larvae and a rapid response to intervention (e.g. 5-10 days of HCD feeding) enable rapid biological readouts and, possibly, large-scale drug screening. We and others have demonstrated that zebrafish are an excellent model to assess the underlying mechanisms of early atherosclerotic lesion development. Furthermore, manipulations of gene expression and/or pharmaceutical interventions to modulate different components involved in lipid metabolism have led to identification of novel lipid metabolism-related mechanisms that regulate angiogenesis. It is plausible that a combination of genetic and chemical interventions and immunological, biochemical and cell biology tools and approaches will continue to provide new mechanistic insights as well as introduce novel therapeutic approaches in the area of lipid metabolism and related diseases.

Acknowledgments

Work in the authors' laboratory is supported by the NIH grants HL093767 (Y.I.M.), HL055798 (Y.I.M.) and HL114734 (L.F.). All authors have read the journal's policy on conflicts of interest and wish to disclose that Y.M. has received an investigator-initiated grant from Merck and is a co-inventor of a patent owned by the University of California for the use of the hypercholesterolemic zebrafish model. Y.M. and L.F. are co-inventors of a patent owned by the University of California related to therapeutic applications of apoA-I binding protein.

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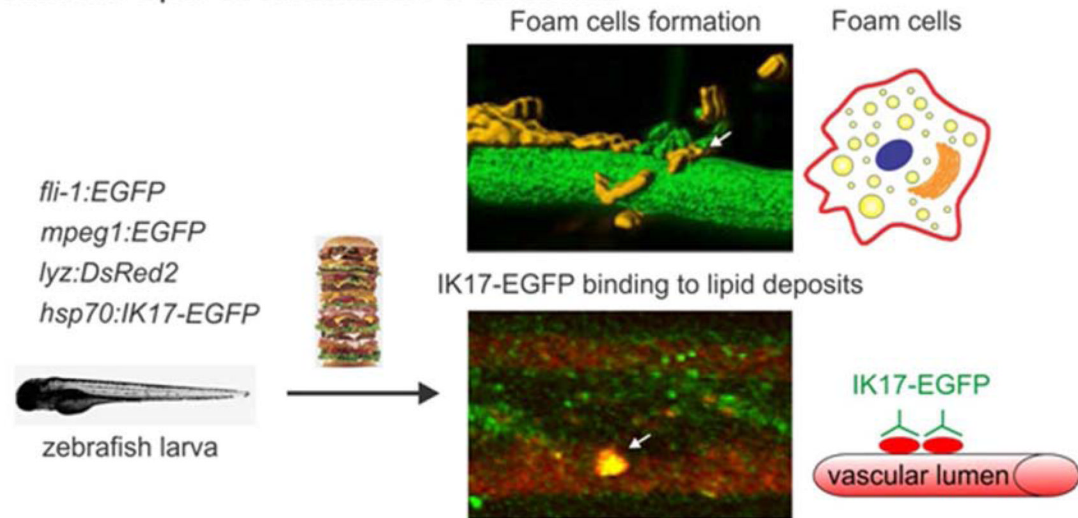

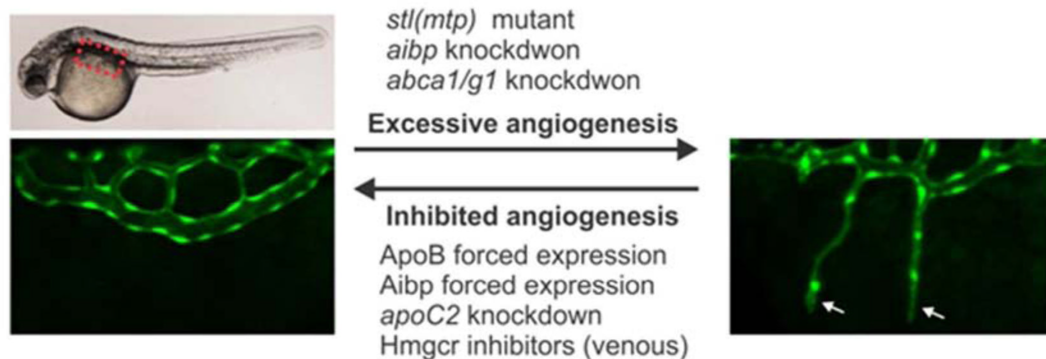

Figure 1. Zebrafish models to study mechanisms relevant to atherosclerosis (A) and angiogenesis (B)

A, Transgenic *fli1:EGFP* zebrafish sport green vasculature, *mpeg1:EGFP* and *lyz:DsRed2* have green macrophages or red myeloid cells (brightest red fluorescence in neutrophils and weaker in monocytes and macrophages), and *hsp70:IK17-EGFP* express the EGFP-tagged anti-malondialdehyde (MDA)-LDL antibody IK17, but only after heat shock (1 hour at 37°C). Feeding either of these transgenic zebrafish a high cholesterol diet initiates vascular processes resembling those that take place during early human atherogenesis. Top image shows a 3D rendering of a blood vessel (green) with lipid-loaded vascular myeloid cells (orange), i.e. foam cells (arrow). Bottom image shows that IK17-EGFP (green) binds to MDA epitopes within vascular lipid deposits (red, because of a red lipid tracer added to the zebrafish diet); the resulting colocalization of green and red displays as yellow (arrow) on the image. **B,** For angiogenesis studies, the genes/proteins that were modified through a mutation, knockdown, overexpression or pharmacologic inhibition, include *mtp* (assembles VLDL; *stl* is an *mtp* loss-of-function mutant), *aibp* (facilitates cholesterol efflux from endothelial cells), *abca1* and *abcg1* (cellular transporters that mediate cholesterol efflux), *apoB* (the LDL protein), *apoC2* (activates lipoprotein lipase), and Hmgcr (enzyme of cholesterol and prenol synthesis pathways). Top-left image shows a zebrafish larva with red box depicting the area where fluorescent images were recorded. Bottom-left is an image of

normal subintestinal veins (SIV), and bottom-right is an image of excessive sprouting in the SIV region. Arrows show two ectopic SIV sprouts.

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Table I
Transgenic zebrafish and fluorescent tracers

Tools	Targets	Applications	References
Transgenic zebrafish			
fli1:EGFP	endothelial cells	angiogenesis, vascular lipid accumulation and inflammation	3, 53
lyz:EGFP *lyz:DsRed2*	myeloid cells	vascular inflammation	3, 25
mpeg1:EGFP	macrophages	vascular inflammation, foam cell formation	24, 26
Lipoprotein/lipid tracers and enzyme substrates			
Dil-LDL	LDL in circulation	LDL distribution in flow	28
cholesteryl BODIPY-C11	CE trafficking, LDL, lipid deposits	vascular lipid accumulation	3, 18
BODIPY-cholesterol NBD-cholesterol	cholesterol trafficking, lipid deposits	vascular lipid accumulation[*], intestinal cholesterol absorption and trafficking	17, 18, 20
BODIPY-C12	fatty acid trafficking, lipid droplets	intestinal fatty acid absorption and trafficking	17
PED6	PLA_2 activity	digestive tract and vascular PLA_2 activity	3, 29

[*] our unpublished observations

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Table II
Zebrafish genes involved in lipid metabolism and angiogenesis

Gene	Function	Intervention	Effect	References
aibp	improves cholesterol efflux	knockdown forced expression	ectopic/excessive angiogenesis inhibition of SeA sprouting	14
abcal	cholesterol efflux	knockdown	ectopic/excessive angiogenesis	14
abcgl	cholesterol efflux	knockdown	ectopic/excessive angiogenesis	14
apoB	LDL protein	forced expression LDL inj ection	inhibition of SIV sprouting	4
apoC2	VLDL/HDL protein; activates LPL	knockdown	inhibition of SeA sprouting	4
mtp	VLDL assembly	mutant (*stl*) knockdown	ectopic/excessive angiogenesis normal angiogenesis	4 6
hmgcr	cholesterol and prenols synthesis	pharmacologic inhibitors	inhibition of venous but not arterial sprouting	77

SeA, segmental artery; SIV, subintestinal vein; LPL, lipoprotein lipase.