

Noncaloric monosaccharides induce excessive sprouting angiogenesis in zebrafish via foxo1a-marcksl1a signal

Reviewed Preprint

v2 • September 9, 2024

Revised by authors

Reviewed Preprint

v1 • April 3, 2024

Xiaoning Wang, Jinxiang Zhao, Jiehuan Xu, Bowen Li, Xia Liu , Gangcai Xie , Xuchu Duan , Dong Liu 

Affiliated Hospital of Nantong University, Nantong Laboratory of Development and Diseases, School of Life Science; Co-innovation Center of Neuroregeneration, Nantong University, Nantong, China • Suqian First Hospital, Suqian, China • Medical School, Nantong University, Nantong, China

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

Artificially sweetened beverages containing noncaloric monosaccharides were suggested as healthier alternatives to sugar-sweetened beverages. Nevertheless, the potential detrimental effects of these noncaloric monosaccharides on blood vessel function remain inadequately understood. We have established a zebrafish model that exhibits significant excessive angiogenesis induced by high glucose, resembling the hyperangiogenic characteristics observed in proliferative diabetic retinopathy (PDR). Utilizing this model, we observed that glucose and noncaloric monosaccharides could induce excessive formation of blood vessels, especially intersegmental vessels (ISVs). The excessively branched vessels were observed to be formed by ectopic activation of quiescent endothelial cells (ECs) into tip cells. Single-cell transcriptomic sequencing analysis of the endothelial cells in the embryos exposed to high glucose revealed an augmented ratio of capillary ECs, proliferating ECs, and a series of upregulated proangiogenic genes. Further analysis and experiments validated that reduced *foxo1a* mediated the excessive angiogenesis induced by monosaccharides via up-regulating the expression of *marcksl1a*. This study has provided new evidence showing the negative effects of noncaloric monosaccharides on the vascular system and the underlying mechanisms.

eLife assessment

This **valuable** study investigates the effect of noncaloric monosaccharides, sugar substitutes that are commonly used by diabetic patients, on angiogenesis in the zebrafish embryo. The authors show that noncaloric monosaccharides and glucose similarly induce excessive blood vessel formation due to the increased formation of tip cells by endothelial cells through the foxo1a-marcksl1a pathway. This **solid** study is of interest for the medical community in charge of the prevention and of the treatment of diabetes and other metabolic diseases.

<https://doi.org/10.7554/eLife.95427.2.sa4>

Introduction

Diabetes mellitus (DM) encompasses a group of chronic diseases characterized by elevated blood glucose levels. Among patients with DM, cardiovascular complications, especially the direct and indirect effects of hyperglycemia on the human vascular network, persist as the primary cause of morbidity and mortality [1]. The harmful effects of hyperglycemia are closely associated with both microvascular and macrovascular complications, including retinopathy, nephropathy, neuropathy, atherosclerosis, ischemic heart disease, stroke, and peripheral artery disease [2, 3]. Endothelial cell dysfunction (ECD) is a systemic pathological state exhibiting disrupted integrity, adhesion, altered proliferation capacity, migration, tube formation, and more [4, 5]. High blood glucose levels over long periods have been demonstrated to be associated with vascular dysfunction both *in vivo* and *in vitro* [6, 7].

The epidemiological evidence has indicated the positive correlation between risks of cardiovascular disease and DM with the consumption of sugar-sweetened beverages (SSBs) and 100% fruit juices, thereby emphasizing the concerns for the adverse effects of sugar intake on cardiometabolic risk factors, regardless of whether the sugar is added or naturally occurring [8–12]. Artificially sweetened beverages (ASBs), which incorporate noncaloric sweeteners or low-caloric additives, have been suggested as healthy alternatives to SSBs [13]. ASBs contain sugar alcohols and polyols, such as sorbitol, xylitol, maltitol, mannitol, erythritol, isomalt, and lactitol. The consumption of ASBs worldwide has gradually increased in recent years [13–15]. However, accumulating studies in the last decade suggested that ASB consumption might be associated with an increased risk of cardiovascular events and diabetes [12, 16–23]. Nevertheless, the underlying mechanisms responsible for these findings remain insufficiently documented.

The zebrafish has been recognized as a valuable animal model for studying metabolic diseases, such as hyperglycemia and diabetic complications, due to its functional conservation in glycol metabolism, pancreas structure, glucose homeostasis, adipose biology, and genetic similarities to mammals [24–26]. The combination of embryonic transparency and transgenic lines, wherein endothelial cells are labeled specifically with fluorescent proteins, facilitates the high-resolution imaging analysis of vascular formation *in vivo*. Immersion of zebrafish in glucose solution has been found to induce diabetic complications, including vascular dysfunction [27–30]. Several recent studies have investigated the effects of high glucose on vascular function in the zebrafish model [30–32]. However, the association between noncaloric monosaccharides and vascular dysfunctions, such as excessive angiogenesis, has not been elucidated. Here, we have successfully established a short-term zebrafish model that exhibits significantly excessive angiogenesis similar to the phenotypes observed in proliferative diabetic retinopathy (PDR) induced by glucose treatment. Using this model, we examined the effects of noncaloric monosaccharides on blood vessel development and investigated the molecular mechanisms. Our results provided new evidence for the negative roles of caloric and noncaloric monosaccharides on vascular development.

Results

Establishment of a short-term model of high-glucose-induced excessive angiogenesis

To establish the short-term zebrafish hyperangiogenic model induced by high-glucose treatment, we immersed the *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos, a transgenic line wherein the endothelial cells were labeled with membrane-bound GFP (Supplementary Figure 1), in glucose solution within a wide range of concentrations and time windows (Supplementary Figure 2). We subsequently measured the glucose concentration in the embryos. We found that the glucose concentration in the embryos treated with high glucose was significantly higher than that in the control group (Supplementary Figure 3). We observed that exposing zebrafish embryos at either 24 hours post fertilization (hpf) or 48 hpf to a 6% D-glucose treatment for a duration exceeding 48 hours led to dramatically increased formation of blood vessels (**Figure 1** [↗](#), Movie 1 and 2), especially intersegmental vessels (ISVs) in the indicated area (**Figure 1b** [↗](#)). The hyperbranched endothelial cells were observed to sprout from existing vessels, including the ISVs, dorsal aorta (DA), and dorsal lateral anastomotic vessel (DLAV) (**Figure 1** [↗](#)) in embryos treated with high glucose.

Additionally, these ectopically branched angiogenic sprouts were not perfused by blood flow. Despite the abnormal vessel formation, no significant developmental defects were observed in these treated embryos when examined under a bright field microscope (Supplementary Figure 4a-c). Moreover, no excessive angiogenic phenotype was observed in the embryos treated with 1%, 2%, 3%, and 4% D-glucose within the corresponding time frame (Supplementary Figure 5).

Fructose and noncaloric monosaccharides induce excessive angiogenesis

Fructose is a ketonic monosaccharide that is an energy source for living organisms. Therefore, our study investigated the potential effects of fructose on vascular dysfunction in comparison to glucose. The result demonstrated fructose-induced excessive angiogenesis in zebrafish embryos (Supplementary Figure 6). Wondering whether the effects of glucose and fructose on vascular development were mediated by metabolic events, we then conducted the same tests by using other noncaloric monosaccharides, including L-glucose, D-mannose, D-ribose, and L-arabinose, which animals could not digest. Interestingly, we observed that all these noncaloric monosaccharides could induce excessive angiogenesis, among which the L-glucose purchased from two companies resulted in a similar phenotype as efficiently as D-glucose did (**Figure 2a-h** [↗](#)). To rule out the effect of osmotic pressure, we treated zebrafish embryos with isotonic disaccharides, including lactose, maltose, and sucrose, which did not cause a significant excessive angiogenic phenotype (Supplementary Figure 4d, h; Supplementary Figure 7). However, higher concentration disaccharide treatment can also cause excessive angiogenesis in zebrafish embryos (Supplementary Figure 8). In addition, we also tested the effects of pyruvic acid but did not observe the excessive angiogenic phenotype in the embryos treated with pyruvic acid solution at 50 nM~50 μ M concentration (Supplementary Figure 9). Furthermore, we examined the arterial and venous identity of the hyperbranched vessels via live imaging analysis of the high glucose-treated *Tg(flt1:YFP::kdr1:ras-mCherry)* line, in which the YFP expression in the artery was relatively higher than that in the vein [33 [↗](#)]. The result revealed that the hyperbranched ectopic vessels comprised arteries and veins (**Figure 2i, j** [↗](#)).

High glucose promotes quiescent endothelial differentiation into tip cells

Given that a high-glucose shock has been observed to induce excessive angiogenesis in 48 hpf embryos, it was hypothesized that the shock might play a crucial role in regulating the differentiation of quiescent endothelial cells (ECs) into active tip cell-like cells and their

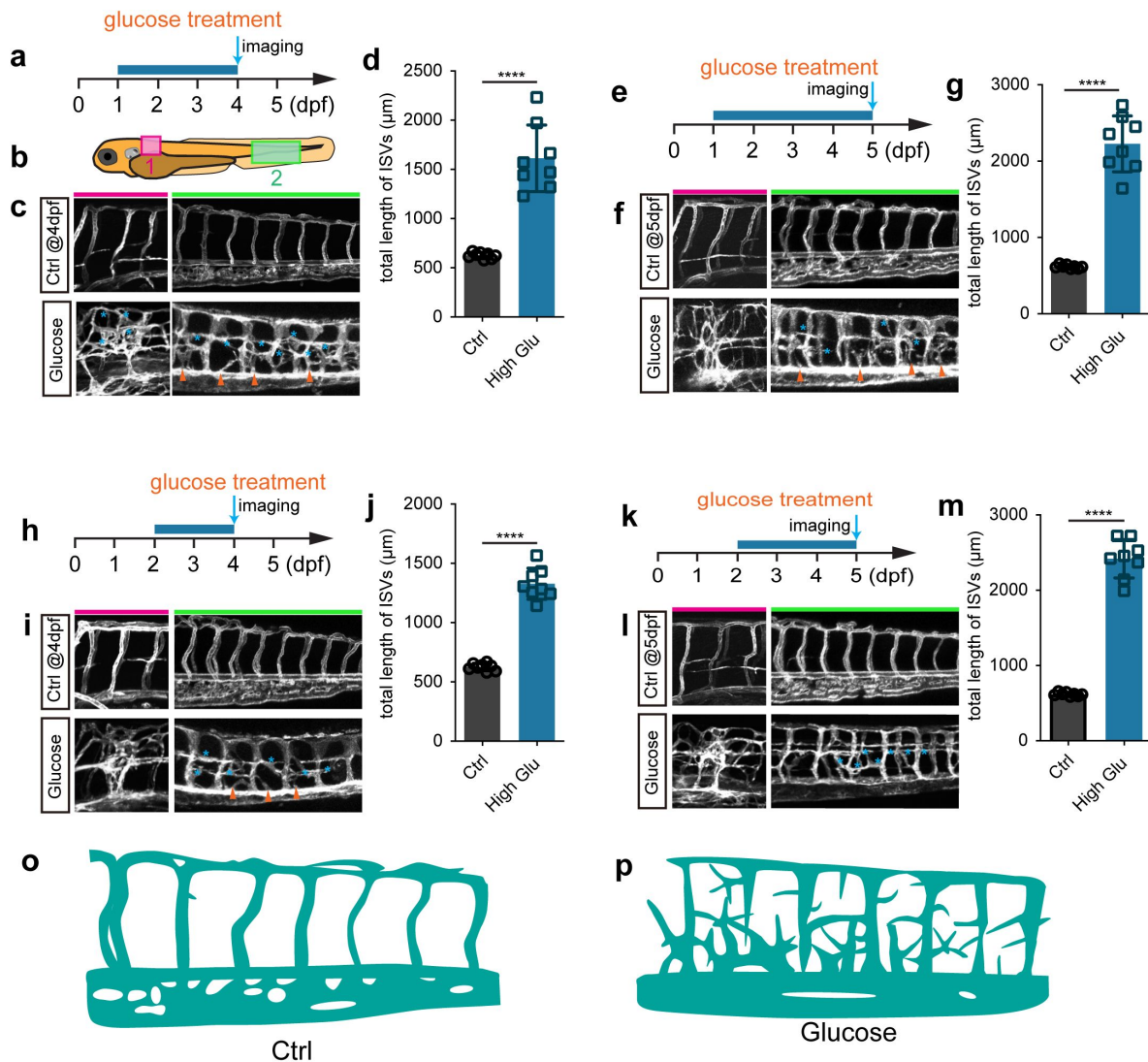


Figure 1

Glucose treatment caused excessive angiogenesis in zebrafish.

a, A diagram showing the glucose treatment time window and imaging time point. **b**, A diagram indicating the imaging positions of the zebrafish embryos. **c**, Confocal imaging analysis of the control and glucose-treated embryos. The red bar indicates position 1; the green bar indicates position 2. Arrowheads indicate the ectopic branching from the dorsal aorta. Stars indicate the ectopic vessels from ISVs and DLAVs. **d**, Statistical analysis of the total length of ISVs in control and glucose-treated embryos. *t*-test, **** $p < 0.0001$. **e**, A diagram showing the glucose treatment time window and imaging time point. **f**, Confocal imaging analysis of the control and glucose-treated embryos. The red bar indicates position 1; the green bar indicates position 2. Arrowheads indicate the ectopic branching from the dorsal aorta. Stars indicate the ectopic vessels from ISVs and DLAVs. **g**, Statistical analysis of the total length of ISVs in control and glucose-treated embryos. *t*-test, **** $p < 0.0001$. **h**, A diagram showing the glucose treatment time window and imaging time point. **i**, Confocal imaging analysis of the control and glucose-treated embryos. The red bar indicates position 1; the green bar indicates position 2. Arrowheads indicate the ectopic branching from the dorsal aorta. Stars indicate the ectopic vessels from ISVs and DLAVs. **j**, Statistical analysis of the total length of ISVs in control and glucose-treated embryos. *t*-test, **** $p < 0.0001$. **k**, A diagram showing the glucose treatment time window and imaging time point. **l**, Confocal imaging analysis of the control and glucose-treated embryos. The red bar indicates position 1; the green bar indicates position 2. Arrowheads indicate the ectopic branching from the dorsal aorta. Stars indicate the ectopic vessels from ISVs and DLAVs. **m**, Statistical analysis of the total length of ISVs in control and glucose-treated embryos. *t*-test, **** $p < 0.0001$. **o**, A diagram showing the blood vessels in position 2 indicated in panel b of control embryos. **p**, A diagram showing the blood vessels in position 2 indicated in panel b of high glucose-treated embryos.

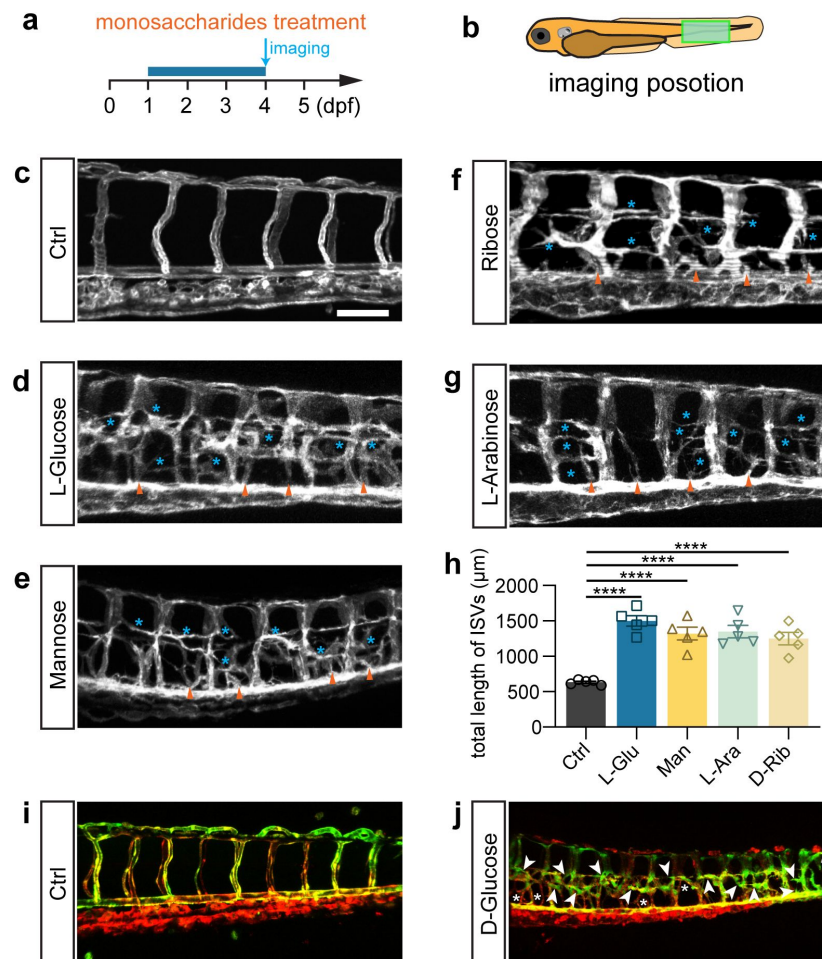


Figure 2

L-glucose and mannose treatment caused excessive angiogenesis as well.

a, A diagram showing the monosaccharides treatment time window and imaging time point. **b**, A diagram indicating the imaging position of the zebrafish embryos. **c-g**, Confocal imaging analysis of the control and monosaccharides, including L-glucose, D-mannose, D-ribose, and L-arabinose, treated embryos. Arrowheads indicate the ectopic branching from the dorsal aorta. Stars indicate the ectopic vessels from ISVs. **h**, Statistical analysis of the total length of ISVs in control and monosaccharides treated embryos. **i-j**, Confocal imaging analysis of the control and glucose-treated embryos. Arrowheads indicate the ectopic branching of arteries. Stars indicate the ectopic branching of veins. *t*-test, **** $p < 0.0001$.

subsequent behaviors. To investigate whether this was the case, we observed the behaviors of these ECs by confocal time-lapse imaging analysis. As shown in the result, in control *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos, no significant activation of tip cells in the angiogenic sprouts was observed in the generated ISVs, DA, and DLAV in the embryos aged from 48 hpf to 5 dpf. Moreover, only a few ECs in established ISVs, DA, and DLAV extended filopodia, which quickly retracted (**Figure 3a-c**, Movie 3). However, many ECs initiated sprouting angiogenesis in the high glucose-treated embryos, extended dynamic filopodia to sense the surroundings, and formed excessive ectopic blood vessels (**Figure 3d-e**, Movie 4). In a snapshot, we observed that some of the ECs protruded long and intricate sprouts simultaneously (**Figure 3f**), and nearly all the ECs within an ISV underwent the outgrowth of filopodia in some extreme cases (**Figure 3g**), suggesting that the high glucose treatment induced the endothelial differentiation into tip cell-like cells. Furthermore, we observed that these outgrowths of the ectopic angiogenic sprouts could establish a connection to the neighboring sprouts and vessels and thereby form complicated vascular structures (**Figure 1c, f, i, l, p**).

Single-cell transcriptomic sequencing analysis of the endothelial cells isolated from glucose-treated embryos

We did a single-cell transcriptomic sequencing analysis to gain more insight into the potential mechanism through which glucose activates the endothelial cells. Due to the limited presence of endothelial cells within the zebrafish embryos, the analysis of these cells poses a challenge. Firstly, we isolated the EGFP-positive cells from control and high glucose-treated embryos. Following the proteolytic dissociation of embryos, the EGFP-positive cells were isolated by fluorescence-activated cell sorting (FACS). Around 300-500 zebrafish embryos were used for the ECs collection for each stage. The isolated ECs were analyzed using a large-scale scRNA-seq (10X Genomics) platform, and the pipeline is illustrated in the diagram (**Figure 4a**). Multiple criteria were applied to select the single cells, including the retention of the genes that were expressed (Unique Molecular Identifiers or UMI larger than 0) in at least 3 individual cells, the selection of cells with the gene expression count falling within the range of 500 to 3000, and the imposition of a threshold wherein the proportion of sequencing reads derived from the mitochondrial genome was limited to less than 5% (Supplementary Figure 10, Supplementary Figure 11, Supplementary Table 1). Ultimately, 6006 endothelial cells were selected for further analysis (Supplementary Table 2).

Through clustering analysis of gene expression, these ECs were categorized into 6 clusters using UMAP. These clusters include cluster 0, which consists of arterial and capillary ECs; cluster1, comprising endocardium; cluster2, consisting of venous and lymphatic ECs; cluster3, comprising arch ECs; cluster4, encompassing proliferating ECs; and cluster5, consisting of vesicle enriched ECs (**Figure 4b**). The endothelial marker gene *cdh5* was expressed in all the clusters (**Figure 4c**). The notch ligand *dlc* was highly expressed in arterial, capillary ECs, and arch ECs (**Figure 4d**). The *dab2* and *prox1* were mainly enriched in venous and lymphatic ECs (**Figure 4e-f**). The *cdk1*, a key player in cell cycle regulation, was specifically expressed in proliferating ECs (**Figure 4h**). It was revealed that the ratio of arterial and capillary ECs and proliferating ECs was increased in the high glucose-treated embryos (**Figure 4i, j**), consistent with the observation that glucose treatment resulted in excessive sprouting angiogenesis of ISVs. In addition, we examined tip cell marker genes in arterial and capillary ECs. The results showed that the expression of *esml*, *cxcr4a*, and *apl* was significantly up-regulated after high glucose treatment (**Figure 3h-k**), consistent with our observation that high glucose treatment induced the endothelial differentiation into tip cell-like cells (**Figure 3f, g**).

We also performed the whole embryo transcriptome sequencing after high D-glucose and L-glucose treatment. We analyzed and compared the differentially expressed genes of control, high D-glucose-treated, and high L-glucose-treated embryos. The results revealed that 1259 and 1074 genes were up-regulated significantly in high D-glucose and high L-glucose treated embryos,

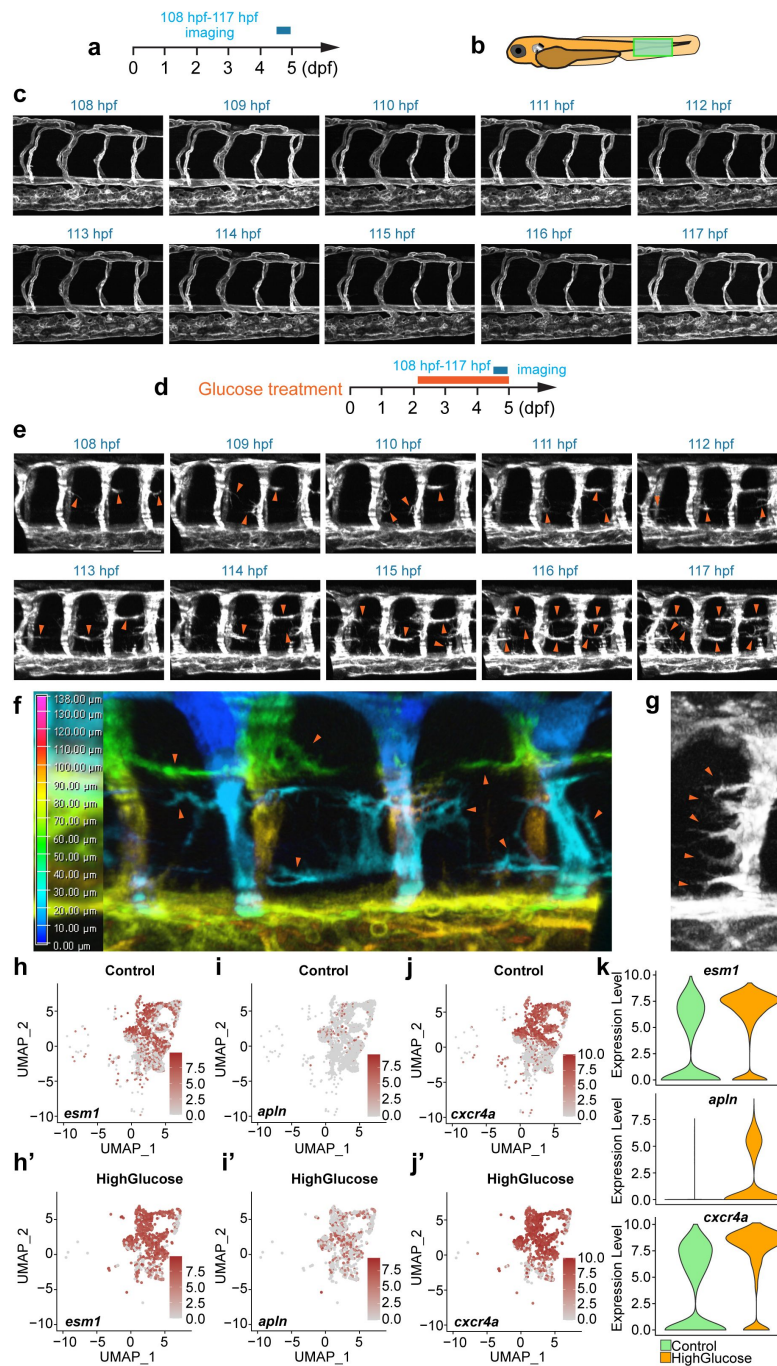


Figure 3

High glucose treatment induced endothelial differentiation into tip cell-like cells.

a, A diagram showing the confocal time-lapse imaging time window. **b**, A diagram indicating the imaging position of the zebrafish embryos. **c**, Confocal time-lapse imaging analysis of blood vessels in control *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **d**, A diagram showing the glucose treatment time window and confocal time-lapse imaging time window. **e**, Confocal time-lapse imaging analysis of blood vessels in glucose-treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Arrowheads indicate the ectopic angiogenic branches. **f**, A snapshot of confocal time-lapse imaging analysis of blood vessels in glucose-treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Z stacks were used to make 3D color projections, where blue represents the most proximal (closest to the viewer), and red represents the most distal (farthest from the viewer). Arrowheads indicate ectopic angiogenic sprouts. **g**, A snapshot of confocal time-lapse imaging analysis of an ISV in glucose-treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Arrowheads indicate ectopic angiogenic sprouts.

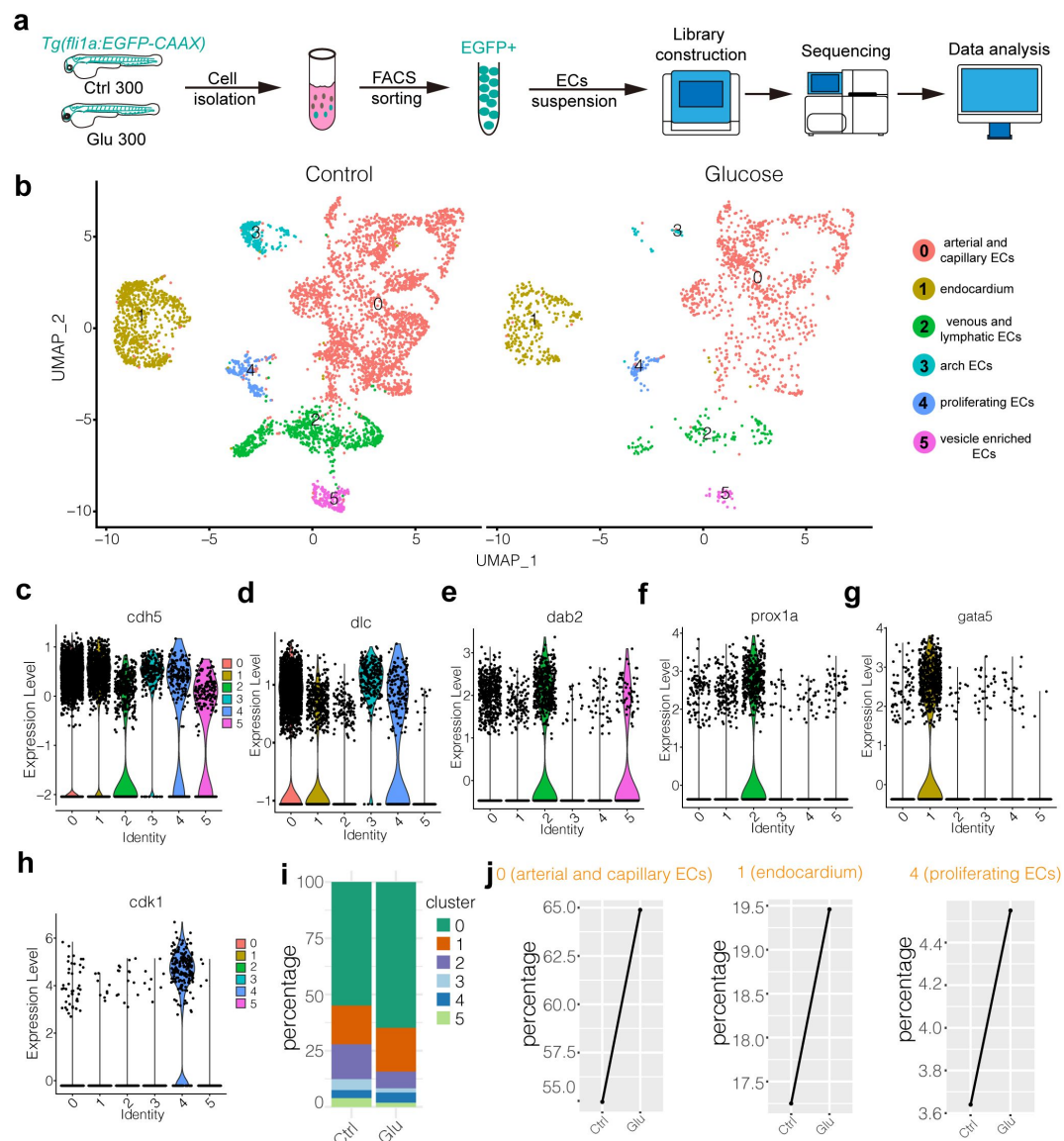


Figure 4
Single-cell transcriptome sequencing analysis of endothelial cells in control and high glucose treated embryos.

a, Schematic diagram of the single-cell sequencing process. 300 embryos in the control group and 300 in the high glucose group were used, and ECs were sorted by GFP fluorescent using FACS technology. **b**, The measured cells were divided into 6 individual clusters based on gene expression profiles using UMAP. **c-h**, The violin plots of some endothelial cell marker genes. **i**, The proportion of ECs in each cluster of control and high glucose groups. **j**, Changes of ECs percentage in arterial and capillary ECs, endocardium, and proliferating ECs of control and high glucose group.

respectively, compared with the control (Supplementary Figure 12). After that, we analyzed the expression of the genes related to metabolic pathways and found significant alteration in the expression of several genes involved in Gluconeogenesis, Glycolysis, and Oxidative phosphorylation (Supplementary Figure 13).

Foxo1a was significantly downregulated in arterial and capillary ECs

To identify the potential molecules responsible for increasing the proportion of arterial and capillary ECs in the embryos treated with glucose, we analyzed and compared the differentially expressed genes (DEGs) in arterial and capillary ECs of control and glucose-treated ECs. The results revealed that 1201 genes were up-regulated and 523 genes were down-regulated significantly (Figure 5a). GO analysis revealed that these DEGs were enriched in several biological processes, including regulation of actin filament organization, blood vessel morphogenesis, development, angiogenesis, etc. (Figure 5b).

Subsequently, we searched for transcription factors among the genes involved in the aforementioned biological processes that might participate in inducing excessive angiogenesis. It has been reported that the loss of function of *foxo1a* led to excessive angiogenesis [34, 35]. Our study also revealed that *foxo1a* was significantly downregulated in arterial and capillary ECs after high glucose treatment compared to the ECs marker gene *pecam1* (Figure 5c-e). The *in situ* hybridization (ISH) experiments further confirmed the decrease in *foxo1a* expression following treatment with high D-glucose and L-glucose (Figure 5f). To verify whether the downregulation of Foxo1a led to excessive angiogenesis in zebrafish embryos, we performed loss-of-function experiments targeting *foxo1a*. AS1842856, a cell-permeable inhibitor reported to block FOXO1 transcription activity [36], was administered to zebrafish embryos at 48 hpf, and the imaging was performed at 72 hpf. The results revealed significantly excessive angiogenesis in AS1842856 treated embryos compared with the control group, consistent with the results obtained from *foxo1a* MO injection (Figure 5g-i).

To further validate that the excessive angiogenesis induced by high glucose was attributed to Foxo1a deficiency, we performed rescue experiments. In detail, *foxo1a* was overexpressed in either whole embryos or ECs, driven by *hsp70l* and *fli1EP* promoter, respectively. We injected the overexpression construct into one-cell stage embryos followed by heat shock at 24 hpf and 48 hpf. The embryos were then treated with high glucose from 48 hpf to 96 hpf (Figure 6a). The results indicated that the gain of function of *foxo1a* in either whole embryos or ECs significantly and partially mitigated the excessive angiogenesis induced by high glucose treatment (Figure 6b-j).

Monosaccharides induced excessive angiogenesis through the *foxo1a-marcks1a* pathway

A previous study has reported that *marcks1a* overexpression in ECs in zebrafish led to a significant increase in filopodia formation, similar to the phenotype we observed in response to high glucose treatment [37]. Our analysis of the single-cell sequencing data revealed a significant upregulation of *marcks1a* in arterial and capillary ECs following high glucose treatment, compared to the ECs marker gene *kdrl* (Figure 7a, b). The real-time qPCR and ISH experiments further confirmed the elevated expression levels of *marcks1a* following high D-glucose and L-glucose treatment (Figure 7c, d). Then, by constructing the transgenic zebrafish line *hsp70l:marcks1a-p2A-mCherry::Tg(fli1a:EGFP-CAAX)^{ntu666}*, we conducted the experiments to overexpress *marcks1a* in zebrafish and subsequently observed the vascular developmental phenotype. After one hour of heat shock at 24 hpf and confocal imaging analysis at 72 hpf, significantly increased blood vessel formation was observed in embryos overexpressing *marcks1a*, compared with the control group (Figure 7e-g).

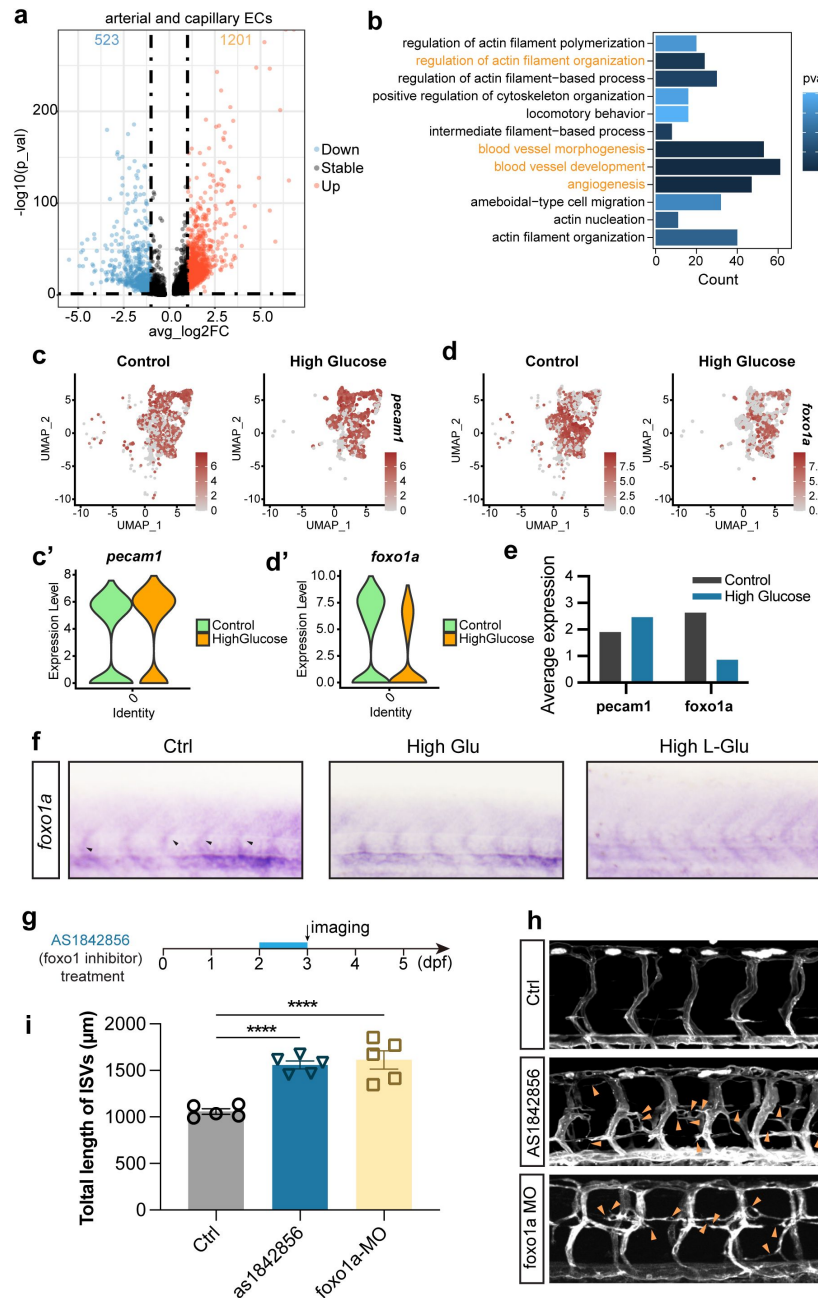


Figure 5

Foxo1a was involved in the excessive angiogenesis induced by high glucose treatment.

a, The volcano plot of differential expression genes in arterial and capillary ECs. The avg_log2FC greater than 1 was considered significant, including 523 down-regulated genes (blue dots) and 1201 up-regulated genes (red dots). **b**, GO analysis of 523 down-regulated genes in arterial and capillary ECs. **c**, The feature plot of ECs marker gene *pecam1* of control and high glucose group in arterial and capillary ECs. **c'**, The violin plot of ECs marker gene *pecam1* of control and high glucose group in arterial and capillary ECs. **d**, The feature plot of gene *foxo1a* of control and high glucose group in arterial and capillary ECs. **d'**, The violin plot of gene *foxo1a* of control and high glucose group in arterial and capillary ECs. **e**, Average expression of gene *pecam1* and *foxo1a* in control and high glucose group. **f**, Whole-mount in situ hybridization analysis of *foxo1a* in control, high glucose, and high L-glucose treated embryos. **g**, A diagram showing the *foxo1* inhibitor treatment time window. **h**, Confocal imaging analysis of control embryos, AS1842856 treated embryos and *foxo1a* MO-injected embryos. Arrowheads indicate ectopic angiogenic sprouts. **i**, Statistical analysis of the total length of ISVs in control embryos, AS1842856 treated embryos, and *foxo1a* MO-injected embryos. *t*-test, **** $p < 0.0001$.

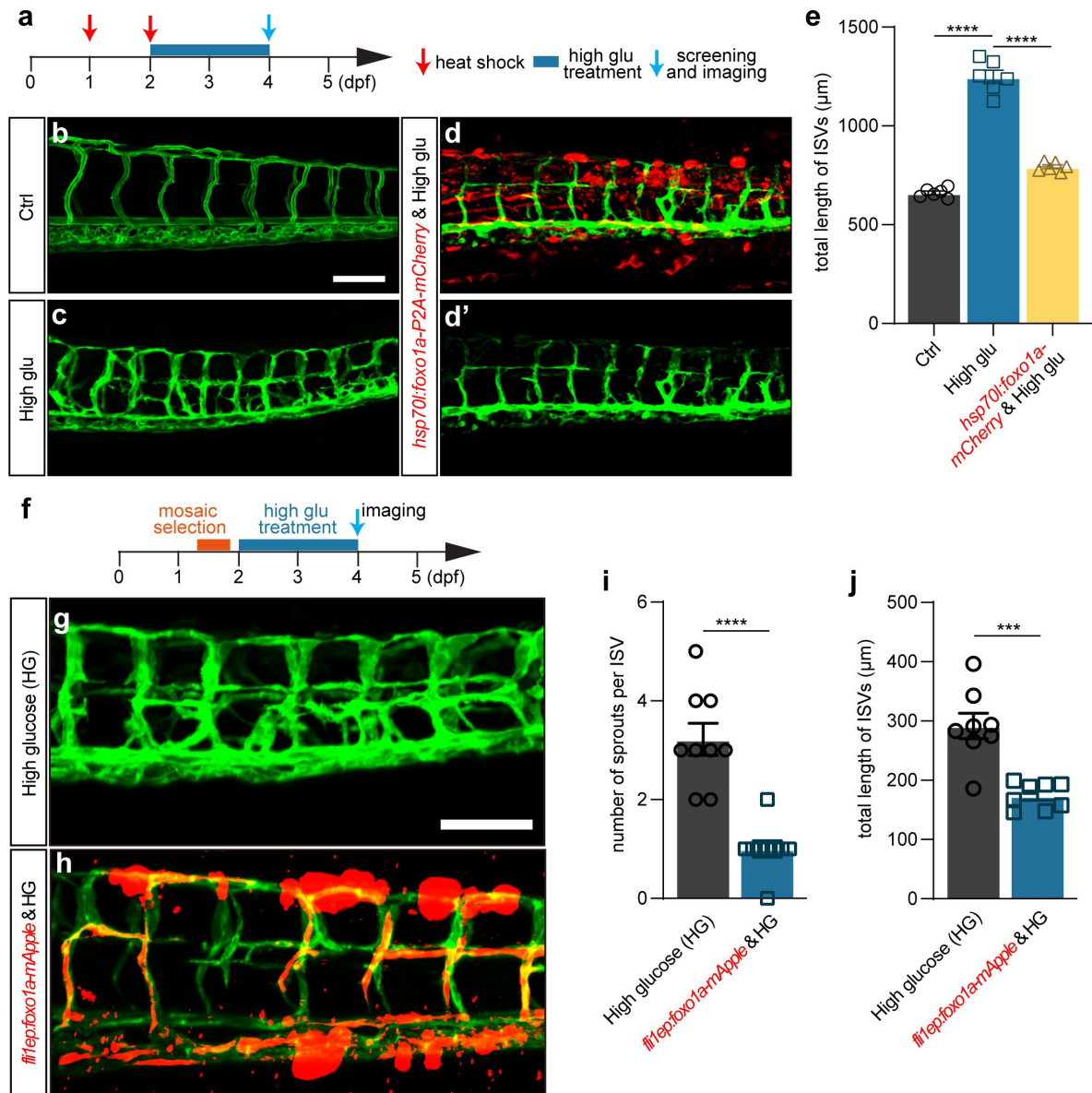


Figure 6

Foxo1a gain of function can partially rescue the excessive angiogenesis induced by high glucose treatment.

a, A diagram showing the high glucose treatment time window and heat shock treated time point. **b**, Confocal imaging analysis of blood vessels in control *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **c**, Confocal imaging analysis of blood vessels in high glucose treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **d-d'**, Confocal imaging analysis of blood vessels in *hsp70l:foxo1a-P2A-mCherry* and high glucose treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **e**, Statistical analysis of the total length of ISVs in control, high glucose-treated, *hsp70l:foxo1a-P2A-mCherry* and high glucose treated embryos, respectively. one-way ANOVA, ****p < 0.0001. **f**, A diagram showing the high glucose treatment time window and imaging time point. **g**, Confocal imaging analysis of blood vessels in high glucose treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **h**, Confocal imaging analysis of blood vessels in *fli1EP:foxo1a-mApple* and high glucose treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **i**, Statistical analysis of the number of sprouts per ISV in high glucose treated embryos, and *fli1EP:foxo1a-mApple* & high glucose treated embryos. *t*-test, ****p < 0.0001. **j**, Statistical analysis of the total length of ISVs in high glucose treated embryos, and *fli1EP:foxo1a-mApple* & high glucose treated embryos. *t*-test, ***p < 0.001.

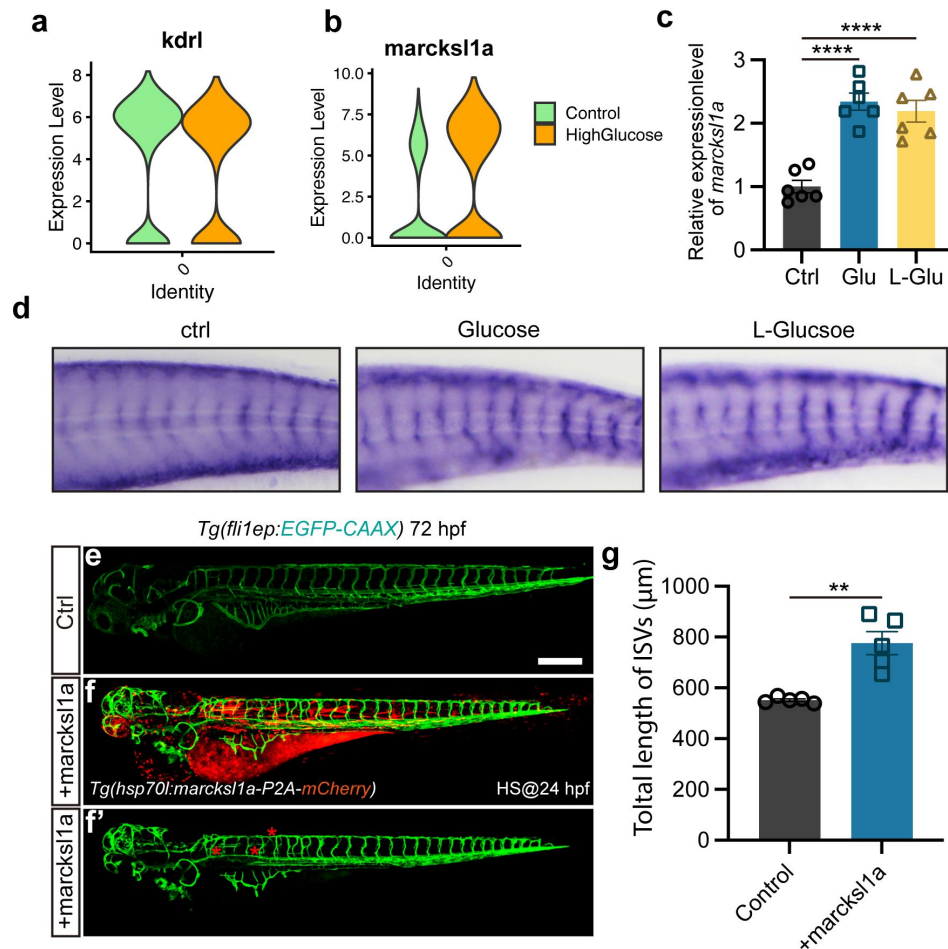


Figure 7

Marcks11a over-expression induced excessive angiogenesis in zebrafish embryos.

a, The violin plot of ECs marker gene *kdr1* of control and high glucose group in arterial and capillary ECs. **b**, The violin plot of gene *marcks11a* of control and high glucose group in arterial and capillary ECs. **c**, Real-time PCR analysis of *marcks11a* expression in control, high glucose, and high L-glucose treated embryos. *t*-test, **** $p < 0.0001$. **d**, Whole-mount in situ hybridization analysis of *marcks11a* in control, high glucose, and high L-glucose treated embryos. **e-f**, Confocal imaging analysis of blood vessels in control and *hsp70l:marcks11a-P2A-mCherry* injected *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **g**, Statistical analysis of the total length of ISVs in control and *hsp70l:marcks11a-P2A-mCherry* injected embryos. *t*-test, ** $p < 0.01$.

Given the results obtained from *marcksl1a* overexpression and loss of function of *foxo1a*, we hypothesized that *marcksl1a* might be a target gene of Foxo1a. Therefore, we investigated the impact of Foxo1 inhibition on *marcksl1a* expression in zebrafish embryos. As expected, qPCR analysis revealed that inhibition of Foxo1a by AS1842856 resulted in the upregulation of *marcksl1a* expression. In contrast, Foxo1a overexpression resulted in the downregulation of *marcksl1a* (Figure 8a-c), which suggested that Foxo1a might negatively regulate *marcksl1a* transcription in zebrafish. To further confirm it, we performed the Chromatin Immunoprecipitation (ChIP) experiment to validate the potential binding interaction between Foxo1 and *marcksl1a*. Since the amino acid sequence and DNA binding motifs of Foxo1 are highly conserved between zebrafish and mice (Supplementary Figure 14), we analyzed the 3 kb promoter region of *marcksl1a* to search the binding site sequence of mouse FOXO1 presented in the JASPAR database. Two candidate binding sites (BS) were found at -265 to -275 (BS1) and -153 to -163 (BS2) nucleotides upstream of the TSS of *marcksl1a* (Figure 8d) and then used for the ChIP-PCR assay detection. The results showed that Foxo1a was enriched in both the predicted binding sites of *marcksl1a* (Figure 8e) in zebrafish. Luciferase reporter assay also indicated that Foxo1a could negatively regulate *marcksl1a* transcription in zebrafish (Figure 8f, g).

Additionally, we microinjected *marcksl1a* MO into the 1-cell stage *Tg(fli1a:EGFP-CAAX)*^{ntu666} embryos, which were then treated with high levels of D-glucose and L-glucose. The findings revealed that the knockdown of Marcksl1a could effectively mitigate the excessive angiogenesis caused by high D-glucose or high L-glucose treatment, resembling the rescue effect observed with VEGFR inhibitor Lenvatinib (Figure 8h-n, Supplementary Figure 15). These results suggested that monosaccharides induced excessive angiogenesis through the Foxo1a-*marcksl1a* pathway in zebrafish embryos.

Discussion

In this study, we successfully established a new zebrafish model with significant excessive angiogenesis, resembling the hyperangiogenic characteristics observed in PDR more closely than previously established models [30, 32]. Seung-Hyun Jung et al. have described a short-term zebrafish model for diabetic retinopathy (DR) induced by high glucose, which exhibited blood vessel defects [30]. However, these defects were limited to the disruption of tight junctions and dilation of hyaloid-retinal vessels [30], without the excessive angiogenesis and vascular blockage observed in PDR and our established model. Additionally, although Kristina Jørgens et al. have observed the hyperbranching of small vessel structures originating from the upper part of ISVs, growing horizontally towards and partially connecting to the neighboring ISVs Field [32], the angiogenic sprouts did not form a more complex structure that was observed in our research.

The excessive development of immature blood vessels represents a significant pathological condition in the progression of DR and nephropathy [38, 39]. Hyperglycemia has been considered one of the most causal factors causing vascular damage, including excessive angiogenesis. However, the exact mechanism through which hyperglycemia impairs the blood vessels is not well determined. We analyzed single-cell transcriptomic sequencing data of the endothelial cells isolated from D-glucose-treated embryos to gain more insights into it. The findings revealed an increased ratio of tip cells and proliferating ECs, accompanied by the altered expression of various angiogenic genes in the ECs of D-glucose-treated embryos.

Foxo1 has been validated to be essential for sustaining the quiescence of endothelial cells, with involvement in metabolism regulation [34, 40]. Moreover, it also plays an important role in diabetic microvascular complications, including DR [41, 42]. Here, by combining the single-cell transcriptomic sequencing data analysis and experimental validation, we identified the transcription factor Foxo1a, which was significantly down-regulated in the embryos treated with high glucose, responsible for the excessive angiogenesis. Additionally, our result further revealed

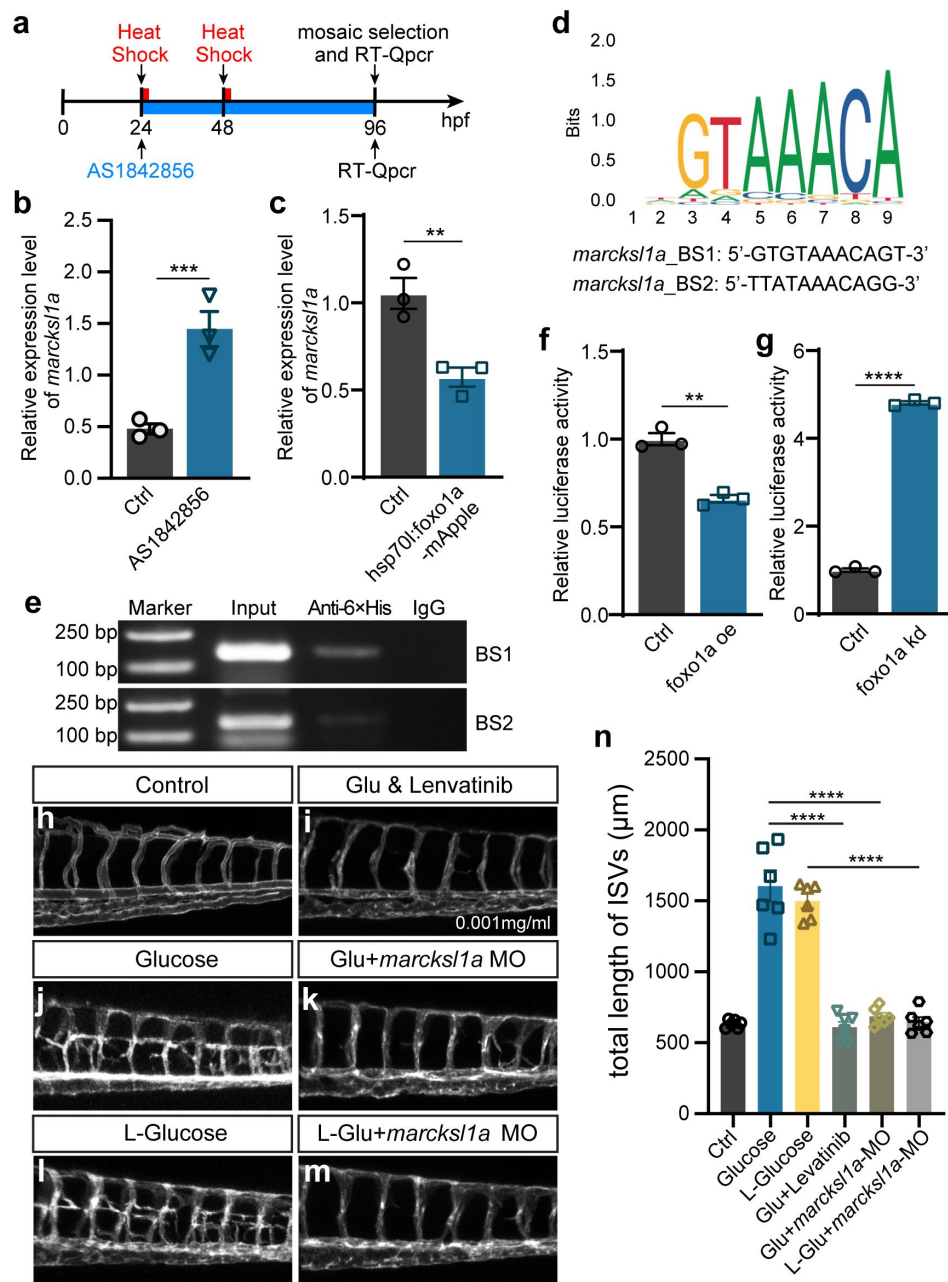


Figure 8

Noncaloric monosaccharides induced excessive angiogenesis through *foxo1a-marcks1a* signal in zebrafish embryos.

a, A diagram showing the Foxo1 inhibitor and heat shock treatment time window. **b**, Real-time PCR analysis of *marcks1a* expression in control and AS1842856 treated embryos. t -test, *** p <0.001. **c**, Real-time PCR analysis of *marcks1a* expression in control and *foxo1a* overexpressed embryos. t -test, ** p <0.01. **d**, A sequence logo of Foxo1 binding sequence presented in the JASPAR database (<https://jaspar.genereg.net/>) and two candidate binding sites at the upstream of transcription start site (TSS) of *marcks1a* in zebrafish. **e**, Results of the ChIP-PCR assay indicated that BS1 and BS2 are Foxo1a-binding sites of *marcks1a* in zebrafish. Input sonicated genomic DNA samples without immunoprecipitation as a positive control. IgG, sonicated, and IgG-immunoprecipitated genomic DNA samples as a negative control. **f-g**, Luciferase reporter activity in *foxo1a* overexpressed or knocked down embryos, respectively. t -test, ** p <0.01, **** p <0.0001. **h-m**, Confocal imaging analysis of blood vessels in control, high glucose, high glucose & Lenvatinib, high glucose+*marcks1a* MO, high L-glucose and high L-glucose+*marcks1a* MO groups. **n**, Statistical analysis of the total length of ISVs in the groups in figure h-m, respectively. one-way ANOVA, **** p <0.0001.

that Foxo1a exerts its regulatory function during this process by down-regulating its target gene *marcksl1a*, regardless of whether the embryos were treated with D-glucose or L-glucose. Taken together, our results suggested that both caloric and noncaloric monosaccharides treatment could lead to excessive angiogenesis by promoting the differentiation of quiescent endothelial cells into tip cells through the *foxo1a-marcksl1a* pathway.

Previous studies have linked the consumption of ASB to the occurrence and development of cardiovascular disease [12, 16–23]. However, the potential mechanisms underlying the association have not been well documented. In recent years, positive associations between ASB and cardiovascular disease have been proposed, possibly due to several plausible factors, including the potential impact of ASBs on central nervous system circuits, gut hormone secretion, and gut microbiota [43–45]. Additionally, it has been hypothesized that the ASBs might stimulate appetite and increase calorie intake [43, 44].

For a long time, there has been considerable debate and conflicting opinions regarding how specific sugars affect the development of type 2 diabetes rather than excess calories per se [46, 47]. In this study, we have provided new evidence indicating that the administration of noncaloric monosaccharides leads to significant excessive angiogenesis, suggesting that the excessive angiogenesis may not be only attributed to the caloric properties. Since excessive angiogenesis is the major pathological feature of diabetic retinopathy and nephropathy, our findings are in support of a possible biological mechanism underlying the positive associations between noncaloric monosaccharides and microvascular complications associated with type 2 diabetes, suggesting that the noncaloric monosaccharides might not be suitable for ASB consumption.

Surprisingly, no notable abnormalities were observed in the vessels of embryos treated with disaccharides, including lactose, maltose, and sucrose, which is consistent with the previous study stating that intakes of sucrose, lactose, and maltose were not significantly associated with the risk of type 2 diabetes [48]. This finding implied that the effects induced by monosaccharides cannot be attributed to the osmotic pressure of the surrounding medium. Furthermore, despite the potential conversion of these disaccharides into monosaccharides, the restricted reaction rate may maintain them within a safe concentration range that is not harmful to the vessels in a short period.

In conclusion, to investigate the effects of monosaccharides on vascular development, we established a zebrafish model by treating the embryos with high concentrations of monosaccharides. Based on this model, we observed significant excessive angiogenesis induced by glucose and noncaloric monosaccharides, initiated by activating the quiescent endothelial cells into proliferating tip cells. The effects of monosaccharides on inducing excessive angiogenesis were then proved to be mediated by the *foxo1a-marcksl1a* pathway. The results have provided novel insights into the roles of noncaloric monosaccharides in human health and the underlying mechanisms.

Materials and methods

Zebrafish

Care and breeding of zebrafish were carried out as previously described [49]. Animal experiments were conducted according to local institutional laws and Chinese law for the Protection of Animals. The following transgenic strains were used: *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* and *Tg(kdr:ras-mCherry)* [33]. Embryos were obtained through natural mating and

maintained at 28.5°C. The stages of zebrafish embryos are defined as previously described [49]. Embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma, P7629) to block pigmentation for further imaging analysis.

Monosaccharides and drug treatment

The D-glucose (Sigma, G7021-100g), L-glucose (Sigma, G5500-1g; J&K, 981195-1g), D-Fructose (Sigma, F0127-100g), L-Rhamnose monohydrate (Aladdin, R108982), D-Sorbitol (Sigma, S1876-100g), D-Mannitol (Sigma, M4125-100g), D-(-)-Ribose (Sigma, V900389-25g), L-(+)-Arabinos (Sigma, V900920-25g), Mannose (Sigma, M2069-25g) and sucrose (Sigma, V900116-500G) were dissolved in E3 solution. Zebrafish embryos at 24 hpf to 48 hpf were placed in 24-well plates (ten embryos per well) and immersed in the solution at the presetting concentrations and time windows. Then, put it in a 28 ° incubator for cultivation. Five days before embryonic development, a stereo fluorescence microscope and a laser confocal microscope were used to observe the changes in blood vessel phenotype. For the drug treatment, the embryos were co-incubated in glucose with Lenvatinib (Selleck, S1164-5MG) at the presetting concentrations and time window. Foxo1 inhibitor AS1842856 (MCE, HY-100596) was dissolved in DMSO and stored at -80° and diluted with E3 solution to 1 µM when used. The same concentration of DMSO was used as a negative control.

Glucose concentration measurement

Glucose concentration in the embryo was measured as described previously [50]. Embryos that developed to 75% epiboly were selected and transferred to 24-well plates (ten embryos per well) and immersed in the solution at the presetting concentrations and time windows. For glucose concentration measurement, embryos (n=20) were transferred to a new 1.5 mL tube, rinsed three times with 1×PBS, and immersed in ice for the following experiments. Discard the PBS as much as possible, embryos homogenized using a hand homogenizer, and centrifuged at 14,000×g for 10 min. 1.5 µL of the supernatant was used to measure the total free-glucose level using a glucometer (Baye, 7600P).

Whole-mount in situ hybridization (WISH)

Whole-mount in situ hybridization and the preparation of antisense RNA probes were performed as described in the previous protocol [51]. Briefly, the *marcksl1a* and *foxo1a* cDNA fragments were cloned with the specific primers listed below using the wild-type embryo (AB) cDNA library. Probes were synthesized using the in vitro DIG-RNA labeling transcription Kit (Roche, 11175025910) with linearized pGEM-T easy vector containing *marcksl1a* or *foxo1a* gene fragment as the templates. Synthesized probes were purified with LiCl (Invitrogen, AM9480) and diluted to 1 ng/µL for hybridization. Zebrafish embryos were collected and fixed with 4% paraformaldehyde (PFA) overnight at 4°C, then dehydrated with methanol gradients and stored at -20° in 100% methanol. The hybridization result was detected with anti-DIG-AP antibody (1:2000, Roche, 11093274910) and NBT/BCIP (1:500, Roche, 11681451001). After hybridization, images of the embryos were captured with an Olympus stereomicroscope MVX10. The primers are listed below:

marcksl1a-probe-forward:5'-AGG ATG GGT GCT CAG TTG AC-3'

marcksl1a-probe-reverse:5'-GCT GGC GTC TCA TTG GTT TC-3'

foxo1a-probe-forward:5'-GCA ACA CAG GAT TTC CCC AC-3'

foxo1a-probe-reverse:5'-CAC AGG TGG CAC TGG AAG G-3'

Single-cell gene expression profile analysis

Cell Ranger 3.0.2 (<https://github.com/10XGenomics/cellranger>) was used to convert the raw sequencing data to a single-cell level gene count matrix. The clustering of single cells and the marker genes in each cluster were analyzed by Seurat 3.0 (<https://satijalab.org/seurat/install.html>) [52]. Several criteria were applied to select the single cells, including only keeping the genes that are expressed (Unique Molecular Identifiers or UMI larger than 0) at least in 3 single cells, selecting single cells with the number of expressed genes at the range between 500 and 3000, and requiring the percentage of sequencing reads on mitochondrial genome being less than 5 percentage. Furthermore, SCTransform method [53] was applied to remove technical variation, and ClusterProfiler [54] was used to do the Gene Ontology enrichment analysis based on the marker genes of each cell cluster. Detailed information about the data processing can be found in this project's source code (<https://github.com/gangcai/ZebEndoimmune>).

Gene expression analysis by quantitative real-time PCR

Total RNA was extracted from zebrafish embryos using TRIzol™ (Invitrogen, 15596026), and stored at -80°. The cDNA was then synthesized using the HiScript II Q RT SuperMix for qPCR Kit (Vazyme, R223-01) according to the manufacturer's instructions. Quantitative PCR was performed in triplicates using the Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Q712-02) on a real-time PCR detection system (StepOne™ Real-Time PCR Systems). The primers used for Real-time PCR analysis are as follows:

ef1a-Qpcr-F:5'-CTT CAA CGC TCA GGT CAT CA-3'

ef1a-Qpcr-R:5'-CGG TCG ATC TTC TCC TTG AG-3'

marcksl1a-Qpcr-F:5'-CCG TGG CTG ATA AAG CCA AT-3'

marcksl1a-Qpcr-R:5'-CTC CCT CCT CCG TTT TTG GG-3'

Transgenic and heat shock

The *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* line was established using a construct *fli1aEP:EGFP-CAAX*, which was generated using multisite Gateway technology, the tol2 kit as previously described [55]. The 5' Entry p5Efl1ep (#31160) purchased from Addgene was originally from Nathan Lawson Lab [56]. Three entry clones and the pDestTol2pA2 destination vector were used to generate the expression construct by LR recombination reaction as described in the Multisite Gateway Manual book. The expression constructs were synthesized by GENEWIZ company. The zebrafish embryos were immersed in a 37 °C water bath for 1 hour for heat shock. Around 75 pg of expression plasmid DNA and 25 pg tol2 transposase mRNA were premixed and microinjected into single-cell fertilized eggs.

Chip-PCR

Embryos injected with *hsp70l:foxo1a-6×His-P2A-mCherry* were collected at 72 hpf after heat shock treatment. According to the manufacturer's instructions, the ChIP-PCR assay was performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore, 3753379). The genomic DNA crossed with Foxo1a protein was immunoprecipitated by using 5 µg Anti-6×His tag antibody (abcam, ab213204). Antibody against IgG was used as a negative control. The semiquantitative PCR was performed with KODfx (TOYOBO, KFX-101) at the following conditions: 94° for 5 min; 35 cycles of 98° for 10 s, 55° for 30 s, 68° for 10 s; 68° for 10 min. The PCR primers used for the predicted binding sites (BS) are as follows:

marcksl1a-BS1-forward:5'-CCC TTT TTC AAA AGT GAG TTT GAG-3'

marcksl1a-BS1-reverse:5'-GGA GCT TCA TCT GCC CCA TT-3'

marcksl1a-BS2-forward:5'-CGG TTT CCA GCT TTC TTC AGA A-3'

marcksl1a-BS2-reverse:5'-TCT CAA ACT CAC TTT TGA AAA AGG G-3'

Luciferase reporter assay

Plasmids of PGL4.10[luc2] and PGL4.74[hRluc/TK] (Promega) were used for luciferase reporter assay. Zebrafish *marcksl1a* promoter fragment with predictive Foxo1a binding site was cloned and inserted into pGL4.10 basic vector by Kpn I and Hind III. The assays for detecting the promoter activity in response to Foxo1a were performed according to the previous study. Briefly, 50 pg of PGL4.10 vectors, 1 pg of PGL4.74 vectors, and 50 pg of *foxo1a* mRNA or 20 pg of *foxo1a* MO were co-injected into wild-type embryos at the one-cell stage. And then, embryos were harvested at 24 hpf to measure their luciferase activity according to the manufacturer's protocols (Promega).

Imaging analysis

For confocal imaging of blood vessels in fluorescence protein labeled transgenic zebrafish embryos, they were anesthetized with egg water/0.16 mg/mL MS222 (Sigma, A5040)/1% PTU and embedded in 0.5-0.8% low melting agarose. Confocal imaging was performed with a Nikon A1R HD25 Confocal Microscope. Analysis was performed using Nikon-NIS-Elements software. The bright field images were acquired with an Olympus DP71 camera on an Olympus stereomicroscope MVX10.

Statistical analysis

Statistical analysis was performed with a student's *t*-test. All data is presented as Mean $\pm$ SEM; $p < 0.05$ was considered statistically significant.

Funding

This study was supported by grants from the National Natural Science Foundation of China (81870359, 2018YFA0801004).

Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and material (data transparency)

All the experimental materials generated in this study are available from the corresponding authors upon reasonable request.

Authors' contributions

Dong Liu, Xuchu Duan, Xia Liu, and Gangcai Xie conceived and designed the experiments and wrote the manuscript. Xiaoning wang, Jinxiang Zhao, Jiehuan Xu, Bowen Li, and Gangcai Xie performed the experiments and analyzed the data. All authors read and approved the final

manuscript.

Ethics approval

All zebrafish experimentation was carried out following the NIH Guidelines for the care and use of laboratory animals (<http://oacu.od.nih.gov/regs/index.htm> ) and ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (Approval ID: 20180905-002).

References

1. Matheus A.S., et al. (2013) **Impact of diabetes on cardiovascular disease: an update** *Int J Hypertens* **2013**
2. Fox C.S., et al. (2004) **Trends in cardiovascular complications of diabetes** *JAMA* **292**:2495–9
3. Rask-Madsen C., King G.L. (2013) **Vascular complications of diabetes: mechanisms of injury and protective factors** *Cell Metab* **17**:20–33
4. Flammer A.J., et al. (2012) **The assessment of endothelial function: from research into clinical practice** *Circulation* **126**:753–67
5. Kolluru G.K., Bir S.C., Kevil C.G. (2012) **Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing** *Int J Vasc Med* **2012**
6. Tesfamariam B., et al. (1990) **Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta** *J Clin Invest* **85**:929–32
7. Ting H.H., et al. (1996) **Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus** *J Clin Invest* **97**:22–8
8. Imamura F., et al. (2015) **Consumption of sugar sweetened beverages, artificially sweetened beverages, and fruit juice and incidence of type 2 diabetes: systematic review, meta-analysis, and estimation of population attributable fraction** *BMJ* **351**
9. Malik V.S (2017) **Sugar sweetened beverages and cardiometabolic health** *Curr Opin Cardiol* **32**:572–579
10. Narain A., Kwok C.S., Mamas M.A. (2016) **Soft drinks and sweetened beverages and the risk of cardiovascular disease and mortality: a systematic review and meta-analysis** *Int J Clin Pract* **70**:791–805
11. Larsson S.C., Akesson A., Wolk A. (2014) **Sweetened beverage consumption is associated with increased risk of stroke in women and men** *J Nutr* **144**:856–60
12. Fung T.T., et al. (2009) **Sweetened beverage consumption and risk of coronary heart disease in women** *Am J Clin Nutr* **89**:1037–42
13. Fakhouri T.H., Kit B.K., Ogden C.L. (2012) **Consumption of diet drinks in the United States, 20092010** *NCHS Data Brief* :1–8
14. Sylvetsky A.C., et al. (2012) **Low-calorie sweetener consumption is increasing in the United States** *Am J Clin Nutr* **96**:640–6
15. Moriconi E., et al. (2020) **Neuroendocrine and Metabolic Effects of Low-Calorie and Non-Calorie Sweeteners** *Front Endocrinol (Lausanne)* **11**
16. de Koning L., et al. (2012) **Sweetened beverage consumption, incident coronary heart disease, and biomarkers of risk in men** *Circulation* **125**:1735–41

17. Fagherazzi G., et al. (2013) **Consumption of artificially and sugar-sweetened beverages and incident type 2 diabetes in the Etude Epidemiologique aupres des femmes de la Mutuelle Generale de l'Education Nationale-European Prospective Investigation into Cancer and Nutrition cohort** *Am J Clin Nutr* **97**:517–23
18. Gardener H., et al. (2012) **Diet soft drink consumption is associated with an increased risk of vascular events in the Northern Manhattan Study** *J Gen Intern Med* **27**:1120–6
19. Drouin-Chartier J.P., et al. (2019) **Changes in Consumption of Sugary Beverages and Artificially Sweetened Beverages and Subsequent Risk of Type 2 Diabetes: Results From Three Large Prospective U.S. Cohorts of Women and Men** *Diabetes Care* **42**:2181–2189
20. de Koning L., et al. (2011) **Sugar-sweetened and artificially sweetened beverage consumption and risk of type 2 diabetes in men** *Am J Clin Nutr* **93**:1321–7
21. Hirahatake K.M., et al. (2019) **Cumulative intake of artificially sweetened and sugar-sweetened beverages and risk of incident type 2 diabetes in young adults: the Coronary Artery Risk Development In Young Adults (CARDIA) Study** *Am J Clin Nutr* **110**:733–741
22. Mossavar-Rahmani Y., et al. (2019) **Artificially Sweetened Beverages and Stroke, Coronary Heart Disease, and All-Cause Mortality in the Women's Health Initiative** *Stroke* **50**:555–562
23. Vyas A., et al. (2015) **Diet drink consumption and the risk of cardiovascular events: a report from the Women's Health Initiative** *J Gen Intern Med* **30**:462–8
24. Zang L., Maddison L.A., Chen W. (2018) **Zebrafish as a Model for Obesity and Diabetes** *Front Cell Dev Biol* **6**
25. Barros T.P., et al. (2008) **Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery** *Br J Pharmacol* **154**:1400–13
26. Elo B., et al. (2007) **Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds** *J Mol Endocrinol* **38**:433–40
27. Gleeson M., Connaughton V., Arneson L.S. (2007) **Induction of hyperglycaemia in zebrafish (Danio rerio) leads to morphological changes in the retina** *Acta Diabetol* **44**:157–63
28. Alvarez Y., et al. (2010) **Predominant cone photoreceptor dysfunction in a hyperglycaemic model of non-proliferative diabetic retinopathy** *Dis Model Mech* **3**:236–45
29. Jorgens K., et al. (2012) **Zebrafish: a model for understanding diabetic complications** *Exp Clin Endocrinol Diabetes* **120**:186–7
30. Jung S.H., et al. (2016) **High glucose-induced changes in hyaloid-retinal vessels during early ocular development of zebrafish: a short-term animal model of diabetic retinopathy** *Br J Pharmacol* **173**:15–26
31. Heckler K., Kroll J. (2017) **Zebrafish as a Model for the Study of Microvascular Complications of Diabetes and Their Mechanisms** *Int J Mol Sci* **18**
32. Jorgens K., et al. (2015) **High tissue glucose alters intersomitic blood vessels in zebrafish via methylglyoxal targeting the VEGF receptor signaling cascade** *Diabetes* **64**:213–25

33. Krueger J., et al. (2011) **Flt1 acts as a negative regulator of tip cell formation and branching morphogenesis in the zebrafish embryo** *Development* **138**:2111–20
34. Wilhelm K., et al. (2016) **FOXO1 couples metabolic activity and growth state in the vascular endothelium** *Nature* **529**:216–20
35. Rudnicki M., et al. (2018) **Endothelial-specific FoxO1 depletion prevents obesity-related disorders by increasing vascular metabolism and growth** *Elife* **7**
36. Zhao W., et al. (2019) **Endothelial CDS2 deficiency causes VEGFA-mediated vascular regression and tumor inhibition** *Cell Res* **29**:895–910
37. Kondrychyn I., et al. (2020) **Marcksl1 modulates endothelial cell mechanoresponse to haemodynamic forces to control blood vessel shape and size** *Nature Communications* **11**
38. Wilkinson-Berka J.L (2004) **Vasoactive factors and diabetic retinopathy: vascular endothelial growth factor, cyclooxygenase-2 and nitric oxide** *Curr Pharm Des* **10**:3331–48
39. Osterby R., Nyberg G. (1987) **New vessel formation in the renal corpuscles in advanced diabetic glomerulopathy** *J Diabet Complications* **1**:122–7
40. Andrade J., et al. (2021) **Control of endothelial quiescence by FOXO-regulated metabolites** *Nat Cell Biol* **23**:413–423
41. Parmar U.M., et al. (2023) **Emerging links between FOXOs and diabetic complications** *Eur J Pharmacol* **960**
42. Behl T., et al. (2022) **Mechanistic insights into the role of FOXO in diabetic retinopathy** *Am J Transl Res* **14**:3584–3602
43. Blundell J.E., Hill A.J. (1986) **Paradoxical effects of an intense sweetener (aspartame) on appetite** *Lancet* **1**:1092–3
44. Pepino M.Y (2015) **Metabolic effects of non-nutritive sweeteners** *Physiol Behav* **152**:450–5
45. Nettleton J.E., Reimer R.A., Shearer J. (2016) **Reshaping the gut microbiota: Impact of low calorie sweeteners and the link to insulin resistance?** *Physiol Behav* **164**:488–493
46. Qi X., Tester R.F. (2020) **Lactose, Maltose, and Sucrose in Health and Disease** *Mol Nutr Food Res* **64**
47. Laville M., Nazare J.A. (2009) **Diabetes, insulin resistance and sugars** *Obes Rev* **10**:24–33
48. Montonen J., et al. (2007) **Consumption of Sweetened Beverages and Intakes of Fructose and Glucose Predict Type 2 Diabetes Occurrence** *The Journal of Nutrition* **137**:1447–1454
49. Wang X., et al. (2016) **MicroRNA-10a/10b represses a novel target gene mib1 to regulate angiogenesis** *Cardiovasc Res* **110**:140–50
50. Da'as S.I., et al. (2023) **The link between glycemic control measures and eye microvascular complications in a clinical cohort of type 2 diabetes with microRNA-223-3p signature** *J Transl Med* **21**

51. Huang Y., et al. (2013) **Nonmuscle myosin II-B (myh10) expression analysis during zebrafish embryonic development** *Gene Expr Patterns* **13**:265–270
52. Butler A., et al. (2018) **Integrating single-cell transcriptomic data across different conditions, technologies, and species** *Nat Biotechnol* **36**:411–420
53. Hafemeister C., Satija R. (2019) **Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression** *Genome Biol* **20**
54. Yu G., et al. (2012) **clusterProfiler: an R package for comparing biological themes among gene clusters** *OMICS* **16**:284–7
55. Kwan K.M., et al. (2007) **The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs** *Dev Dyn* **236**:3088–99
56. Villefranc J.A., Amigo J., Lawson N.D. (2007) **Gateway compatible vectors for analysis of gene function in the zebrafish** *Dev Dyn* **236**:3077–87

Editors

Reviewing Editor

Daniel Henrion

University of Angers, Angers, France

Senior Editor

Matthias Barton

University of Zurich, Zurich, Switzerland

Reviewer #1 (Public Review):

Dong Liu et al. successfully established a short-term zebrafish model by treating the embryos with high concentrations of monosaccharides, resembling the hyperangiogenic characteristics observed in proliferative diabetic retinopathy. The authors found that excessive angiogenesis induced by glucose and noncaloric monosaccharides can be achieved by activating the quiescent endothelial cells into proliferating tip cells. Importantly, the authors further confirmed the effects of monosaccharides on inducing excessive angiogenesis were mediated by the foxo1a-marcksl1a pathway. These results demonstrate the potentially detrimental effects of the noncaloric monosaccharides on blood vessel function and provided novel insights into the underlying mechanisms.

<https://doi.org/10.7554/eLife.95427.2.sa3>

Reviewer #2 (Public Review):

In the manuscript Liu et al. observed that glucose and noncaloric monosaccharides can prompt an excessive formation of blood vessels, particularly intersegmental vessels (ISVs). They propose that these branched vessels arise from the ectopic activation of quiescent endothelial cells (ECs) into tip cells. Moreover, through single-cell transcriptome sequencing analysis of embryonic endothelial cells exposed to glucose, they noted an increased proportion of arterial and capillary endothelial cells, proliferative endothelial cells, along with a series upregulated genes in categories of blood vessel morphogenesis, development,

and pro-angiogenesis. The authors provide evidence suggesting that caloric and noncaloric monosaccharides (NMS) induce excessive angiogenesis via the Foxo1a-Marcksl1a pathway.

<https://doi.org/10.7554/eLife.95427.2.sa2>

Reviewer #3 (Public Review):

The authors have investigated the effect of noncaloric monosaccharides on angiogenesis in the zebra fish embryo. These compounds are used as substitutes of sugars to sweeten beverages and they are commonly used by diabetic patients. The authors show that noncaloric monosaccharides and glucose similarly induce excessive blood vessels formation due to increased formation of tip cells by endothelial cells. The authors show that this excessive angiogenesis involved the foxo1a-marcksl1a pathway.

A limitation of the study is that the mechanism of angiogenesis in the retinal circulation and in peripheral vasculature is certainly different.

This result suggests that these noncaloric monosaccharides share common side effects with glucose. Consequently, more caution should be taken as regard to the use of these artificial sweeteners. This work is of interest for a better management of diabetes.

<https://doi.org/10.7554/eLife.95427.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1:

(1) The authors claimed that they examined the arterial and venous identity of the hyperbranched vessels via live imaging analysis of the high glucose-treated Tg(flt1:YFP::kdrl:ras-mCherry) line, and revealed that the hyperbranched ectopic vessels comprised arteries and veins. That's good, of course. However, there are no relevant results in Figure 2. Please revise it.

Thank you very much for the suggestion. We've added this part of the results in Figure 2i and j.

(2) In Figures 3f and 3g, some of the ECs protruded long and intricate sprouts, and nearly all the ECs within an ISV underwent the outgrowth of filopodia in some extreme cases (Figure 3g), suggesting that the high glucose treatment induced the endothelial differentiation into tip cell-like cells. The findings are surprising and interesting. In order to further confirm the author's conclusion, in situ hybridization experiments are more appropriate to show the expression changes of tip cell-like cell marker genes in the high glucose-treated embryos.

Thank you very much for your constructive suggestions. We have performed the analysis of single-cell RNA-seq data, and the results showed that the tip cell marker genes such as *esm1*, *apln*, and *cxcr4a* were significantly up-regulated in arterial and capillary ECs after high glucose treatment. The results were integrated into Figure 3 of the revised manuscript.

(3) Embryos treated with AS1842856 or injected with foxo1a-MO exhibited excessive angiogenesis (Figure 5g-i), suggesting the transcription activity of foxo1 is required to

maintain the quiescent state of endothelial cells. Did the downregulation of foxo1a lead to the differentiation of endothelial cells into tip-cell-like cells?

Thank you very much for the question. We examined our results carefully and marked these tip cell-like cells with arrow heads in Figure 5h of the revised manuscript.

(4) Foxo1a was significantly downregulated in arterial and capillary ECs after high glucose treatment (Figure 5c-e). More importantly, whether overexpression of foxo1a in the high glucose-treated embryos could eliminate the hyperangiogenic characteristics?

Thank you for the great questions. We performed rescue experiments, and the results suggested that the overexpression of *foxo1a* partially mitigated the excessive angiogenesis induced by high glucose treatment. These results were integrated into Figure 6 of the revised manuscript.

(5) The authors' results found that foxo1a was enriched in both the predicted binding sites of marcksl1a by ChIP-PCR experiments (Figure 7d). This result is reliable. However, whether these two sites are important for marcksl1a gene transcription needs to be confirmed by relevant experiments, such as luciferase reporter assays.

We've performed the luciferase reporter assays and added these data to Figure 8f and g.

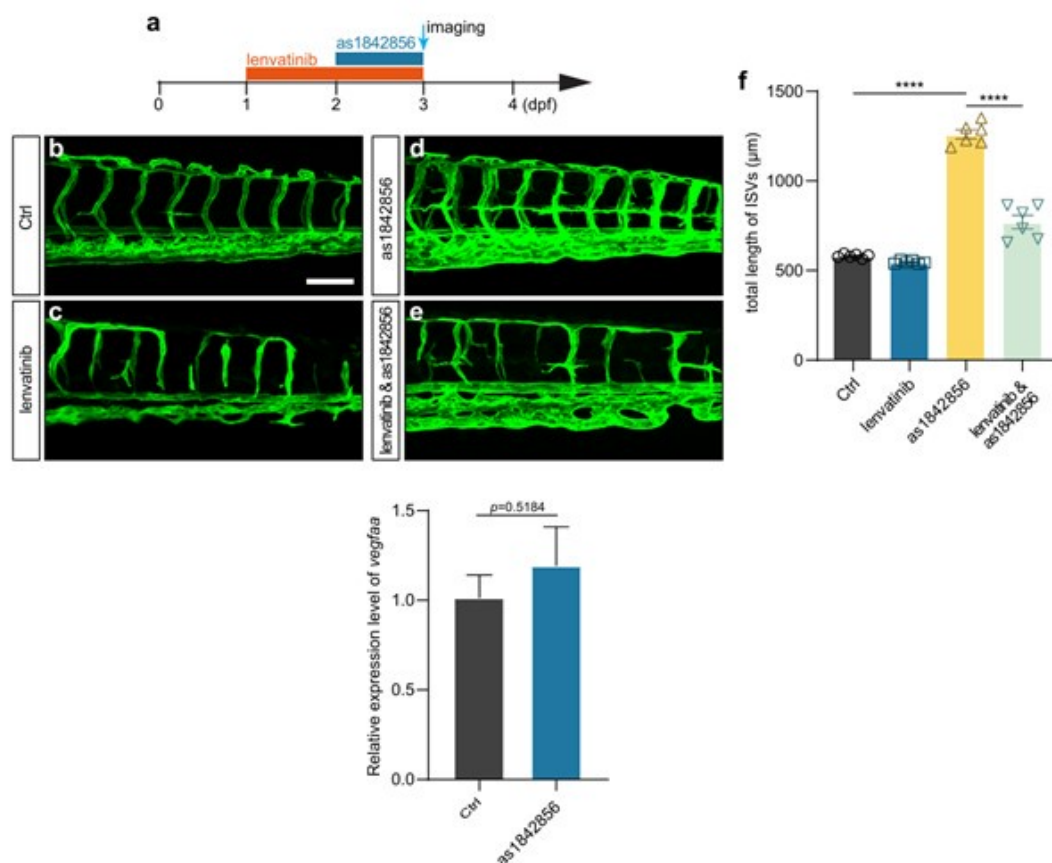
Reviewer #2:

Suggested major experiments:

(1) A previous study (Jorgens et al., Diabetes 64, 2015) reported that high tissue glucose levels increased reactive dicarbonyl methylglyoxal (MG) concentrations in zebrafish embryos and triggered the formation of hyperbranched ISVs. Additionally, they illustrated that MG induced the vascular hyperbranching phenotype via enhancing phosphorylated VEGFR and pAKT signaling cascade. The authors must examine whether both pVEGFR and pAKT are increased in noncaloric monosaccharide (NMS)-treated embryos. The authors need also to test the crosstalks between VEGFR/AKT signaling and foxo1a-Marcksl1a pathway in glucose or NMS-treated embryos.

Thank you very much for your suggestion. We treated the embryos with AS1842856 (*foxo1* inhibitor) and Lenvatinib (VEGFR inhibitor), and the results showed that Lenvatinib treatment attenuated the excessive angiogenesis induced by *foxo1* inhibition. We also examined the expression level of *vegfaa* after AS1842856 treatment; the results suggested that *foxo1* inhibition did not affect the expression of *vegfaa*.

Author response image 1.



(2) In this manuscript, the authors performed single endothelial cell sequencing in glucose-treated embryos, and found reduced *foxo1a* expression and upregulated *marcksl1a*. Based on these data, the authors demonstrated that glucose and NMS-induced excessive angiogenesis through the *foxo1a-marcksl1a* pathway. The authors must conduct endothelial scRNA-seq in NMS-treated embryos, and analyze and compare the datasets with scRNA-seq datasets from glucose-treated endothelial cells, considering the focus of the paper. In addition, ASBs have been suggested as healthy alternatives to sugar-sweetened beverages. The authors also need to examine carefully whether metabolic gene programs are altered in glucose-treated endothelial cells, which was mentioned in Jorgens et al paper.

Thank you very much for your constructive suggestions. We have performed the whole embryo transcriptome sequencing after high D-glucose and L-glucose treatment. We analyzed and compared the differentially expressed genes of control, high D-glucose-treated, and high L-glucose-treated embryos. The results revealed that 1259 and 1074 genes were up-regulated significantly in high D-glucose and high L-glucose treated embryos, respectively, compared with control.

We also analyzed some metabolic-related genes and found that some genes involved in gluconeogenesis, glycolysis, and oxidative phosphorylation were significantly changed. The results were integrated into supplementary Figure12 and 13 of the revised manuscript.

(3) Glucose or NMS treatments induce the hyperbranched endothelial vessels from the dorsal aorta and ISVs but not cardinal veins. In Figure 4i, the arterial and capillary cell population is increased in glucose-treated embryos, but the venous cell population seems to be reduced. The authors need to check whether arterial/venous differentiation and proliferation are affected in glucose- and NMS-treated embryos.

Thank you for your suggestions. We examined arterial/venous differentiation based on Tg(*flt1BAC:YFP::kdr1:ras-mCherry*) zebrafish line, in which the YFP is mainly expressed in arterial Endothelial cells. We found the endothelial cells of excessively formed blood vessels induced by high glucose treatment are mainly arterial (Figure 2j). This might explain why the arterial and capillary cell population was increased in glucose-treated embryos.

(4) The manuscript proposes that excessively branched vessels within ISVs arise from the ectopic activation of quiescent endothelial cells (ECs) into tip cells. To confirm this process, the authors need to detect some specific tip cell markers to demonstrate their ectopic activation.

Thank you for your constructive suggestions. We have performed the analysis of single-cell RNA-seq data, and the results showed that the tip cell marker genes such as *esm1*, *apln*, and *cxcr4a* were significantly up-regulated in arterial and capillary ECs after high glucose treatment. The results were integrated into Figure 3 of the revised manuscript.

(5) Disaccharides such as lactose, maltose, and sucrose did not exhibit a notable induction of excessive angiogenic phenotype. However, the specific treatment concentrations utilized in the study were not delineated. Therefore, further investigation is warranted to determine whether increased disaccharide concentrations can cause vascular hyperbranching phenotype.

Thank you very much for the suggestions. We've described the concentrations of monosaccharides and disaccharides in the materials and methods section of the revised manuscript. Following the suggestion, we treated zebrafish embryos with a higher concentration of the disaccharide. The results showed that higher concentrations of disaccharide treatment also caused excessive angiogenesis in zebrafish embryos. These results were integrated into supplementary Figure 8 of the revised manuscript.

(6) The authors claim that glucose and NMS (such as L-glucose) induce excessive angiogenesis through the foxo1a-marcks1a pathway. Following exposure to elevated glucose levels, a substantial down-regulation of foxo1a was observed in arterial and capillary endothelial cells. This down-regulation led to the release of foxo1a inhibition on marcks1a, subsequently resulting in an augmented expression of marcks1a and the manifestation of a vascular phenotype. Consequently, it is imperative to investigate whether the foxo1a overexpression can attenuate marcks1a expression and mitigate the vascular phenotype induced by monosaccharides. Sufficient data support is needed for the conclusion that monosaccharides induce angiogenesis via the foxo1a-marcks1a pathway.

Thank you very much for your constructive suggestions.

We confirmed the expression of *marcks1a* in foxo1a-overexpressed embryos. The results indicated that foxo1a overexpression significantly attenuated *marcks1a* expression. The results were integrated into Figure 8c. We also performed the rescue experiments, which indicated that overexpression of *foxo1a* partially mitigated the excessive angiogenesis

induced by high glucose treatment. These results were integrated into Figure 6 of the revised manuscript.

Minor corrections:

(1) Figure 2i, j has no corresponding graphs.

We've made the change in Figure 2.

(2) Figure 2h has no vertical coordinates.

We've made the change in Figure 2.

(3) All Figures should be referenced within the manuscript.

We've checked our manuscript carefully and made the corrections.

(4) The concentrations of monosaccharides and disaccharides employed in this study must be distinctly elucidated within the manuscript and annotated using the internationally recognized unit notation.

We've checked our manuscript carefully and described the concentrations of monosaccharides and disaccharides in the revised materials and methods section.

Reviewer #3:

(1) A possible limitation of the study is that the mechanism leading to angiogenesis in the retinal circulation and in peripheral vasculature is certainly different as diabetes is associated with excessive angiogenesis in the retina and a defect in angiogenesis in the peripheral circulation as shown by a reduced post-ischemic revascularization (see Silvestre et al.: DOI: 10.1152/physrev.00006.2013).

Thank you very much for your suggestions. As you said, the peripheral blood vessel model in this study does not fully represent individuals with diabetic retinopathy, which is a limitation. However, from a specific view, the phenotype and mechanism of excessive angiogenesis of peripheral blood vessels in the high glucose model may provide a reference for excessive angiogenesis in the retina; they might have similar etiology and regulation mechanisms in excessive angiogenesis.

(2) Another limitation is that angiogenesis in the embryo is not fully representative of the excessive angiogenesis observed in the diabetic retinal circulation. It would be of interest to analyse the retinal vascular tree in adult fish submitted to high glucose and to ASB.

In our future study, we will try to observe the angiogenesis phenotype in the diabetic retina and improve the disease model.

(3) Line 52: "Endothelial cell dysfunction (ECD)" instead of "Endothelial dysfunction (ECD)".

We've made the correction in the revised manuscript.

(4) The authors should elaborate more on the observation showing that L-glucose, D-mannose, D-ribose, and L-arabinose, which could not be digested by animals, also induce excessive angiogenesis. Is the effect indirect?

In the current manuscript, we conducted an in vivo live imaging analysis to show the phenotype of excessive angiogenesis caused by those noncaloric monosaccharides. However, we did not find differences in the phenotypes of embryos treated with noncaloric and caloric monosaccharides. Therefore, we supposed that the mechanisms underlying the phenotypes were similar. The effect might be indirect.

<https://doi.org/10.7554/eLife.95427.2.sa0>