1 **Title: Noncaloric monosaccharides induce excessive sprouting angiogenesis in zebrafish**

2 **via foxo1a-marcksl1a signal**

3 **Running title: Noncaloric Monosaccharides Induce Excessive Angiogenesis**

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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. Presently, 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 foxo1a mediated the excessive angiogenesis induced by monosaccharides by down-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.

Key words: noncaloric monosaccharides; endothelial cell activation; foxo1a; marcksl1a; zebrafish

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 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

66   [12, 16-23]. Nevertheless, the underlying mechanisms responsible for these findings remain

67   insufficiently documented.

68   The zebrafish has been recognized as a valuable animal model for studying metabolic

69   diseases, such as hyperglycemia and diabetic complications, due to its functional

70   conservation in glycol metabolism, pancreas structure, glucose homeostasis, adipose biology,

71   and genetic similarities to mammals [24-26]. The combination of embryonic transparency

72   and transgenic lines, wherein endothelial cells are labeled specifically with fluorescent

73   proteins, facilitates the high-resolution imaging analysis of vascular formation *in vivo*.

74   Immersion of zebrafish in glucose solution has been found to induce diabetic complications,

75   including vascular dysfunction [27-30]. Several recent studies have investigated the effects of

76   high glucose on vascular function in the zebrafish model [30-32]. However, the association

77   between noncaloric monosaccharides and vascular dysfunctions, such as excessive

78   angiogenesis, has not been elucidated. Here, we have successfully established a short-term

79   zebrafish model that exhibits significantly excessive angiogenesis similar to the phenotypes

80   observed in proliferative diabetic retinopathy (PDR) induced by glucose treatment. Using this

81   model, we examined the effects of noncaloric monosaccharides on blood vessel development

82   and investigated the molecular mechanisms. Our results provided new evidence for the

83   negative roles of caloric and noncaloric monosaccharides on vascular development.

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85

86   **Results**

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

88   To establish the short-term zebrafish hyperangiogenenic model induced by high-glucose

89   treatment, we immersed the *Tg(fli1aEP:EGFP-CAAX)*[ntu666] embryos, a transgenic line

90   wherein the endothelial cells were labeled with membrane-bound GFP (Supplementary

91   Figure 1), in glucose solution within a wide range of concentrations and time windows

92   (Supplementary Figure 2). We subsequently measured the glucose concentration in the

93   embryos and found that the glucose concentration in the embryos treated with high glucose

94   was significantly higher than that in the control group (Supplementary Figure 3). We

95   observed that exposing zebrafish embryos at either 24 hours post fertilization (hpf) or 48 hpf

96   to a 6% D-glucose treatment for a duration exceeding 48 hours led to dramatically increased

97   formation of blood vessels (Figure 1, Movie 1 and 2), especially intersegmental vessels

98   (ISVs) in the indicated area (Figure 1b). The hyperbranched endothelial cells were observed

99   to sprout from existing vessels, including the ISVs, dorsal aorta (DA), and dorsal lateral

100   anastomotic vessel (DLAV) (Figure 1) in embryos treated with high glucose. Additionally,

101   these ectopically branched angiogenic sprouts were not perfused by blood flow. Despite the

102   abnormal vessel formation, no significant developmental defects were observed in these

103   treated embryos when examined under a bright field microscope (Supplementary Figure 4a-

104   c). Moreover, no excessive angiogenic phenotype was observed in the embryos treated with

105   1%, 2%, 3%, and 4% D-glucose within the corresponding time frame (Supplementary Figure

106   5). In addition, 6% sucrose treatment at the same condition did not lead to any noticeable

107   abnormal phenotype of vessels (Supplementary Figure 4d-h).

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 could not be digested by animals. 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). However, the disaccharides, including lactose, maltose, and sucrose, which were also tested, did not cause significant excessive angiogenic phenotype (Supplementary Figure 4e, f; Supplementary Figure 7). 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 μm concentration (Supplementary Figure 8). Furthermore, we 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, 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

129   Given that a high-glucose shock has been observed to induce excessive angiogenesis in 48

130   hpf embryos, it was hypothesized that the shock might play a crucial role in regulating the

131   differentiation of quiescent endothelial cells (ECs) into active tip cell-like cells and their

132   subsequent behaviors. To investigate whether this was the case, we observed the behaviors of

133   these ECs by confocal time-lapse imaging analysis. As shown in the result, in the control

134   *Tg(fli1aEP:EGFP-CAAX)[ntu666]* embryos, no significant activation of tip cells in the

135   angiogenic sprouts was observed in the generated ISVs, DA, and DLAV in the embryos aged

136   from 48 hpf to 5 dpf. Moreover, only a few ECs in established ISVs, DA, and DLAV

137   extended filopodia, which quickly retracted (Figure 3a-c, Movie 3). However, many ECs

138   initiated sprouting angiogenesis in the high glucose-treated embryos, extended dynamic

139   filopodia to sense the surroundings, and formed excessive ectopic blood vessels (Figure 3d-e,

140   Movie 4). In a snapshot, we observed that some of the ECs protruded long and intricate

141   sprouts simultaneously (Figure 3f), and nearly all the ECs within an ISV underwent the

142   outgrowth of filopodia in some extreme cases (Figure 3g), suggesting that the high glucose

143   treatment induced the endothelial differentiation into tip cell-like cells. Furthermore, we

144   observed that these outgrowths of the ectopic angiogenic sprouts could establish a connection

145   to the neighboring sprouts and vessels and thereby form complicated vascular structures

146   (Figure 1c, f, i, l, p).

147   **Single-cell transcriptomic sequencing analysis of the endothelial cells isolated from**

148   **glucose-treated embryos**

149   We did a single-cell transcriptomic sequencing analysis to gain more insight into the

150   potential mechanism through which glucose activates the endothelial cells. Due to the limited

151   presence of endothelial cells within the zebrafish embryos, the analysis of these cells poses a

152   challenge. Firstly, we isolated the EGFP-positive cells from control and high glucose-treated

153   embryos. Following the proteolytic dissociation of embryos, the EGFP-positive cells were

154   isolated by fluorescence-activated cell sorting (FACS). Around 300-500 zebrafish embryos

155   were used for the ECs collection for each stage. The isolated ECs were analyzed using a

156   large-scale scRNA-seq (10X Genomics) platform, and the pipeline is illustrated in the

157   diagram (Figure 4a). Multiple criteria were applied to select the single cells, including the

158   retention of the genes that were expressed (Unique Molecular Identifiers or UMI larger than

159   0) in at least 3 individual cells, the selection of cells with the gene expression count falling

160   within the range of 500 to 3000, and the imposition of a threshold wherein the proportion of

161   sequencing reads derived from the mitochondrial genome was limited to less than 5%

162   (Supplementary Figure 9). Ultimately, 6051 endothelial cells were selected for further

163   analysis (Supplementary Figure 10, Supplementary Table 1).

164   Through clustering analysis of gene expression, these ECs were categorized into 6 clusters

165   using UMAP. These clusters include cluster 0, which consists of arterial and capillary ECs;

166   cluster1, comprising endocardium; cluster2, consisting of venous and lymphatic ECs;

167   cluster3, comprising arch ECs; cluster4, encompassing proliferating ECs; and cluster5,

168   consisting of vesicle enriched ECs (Figure 4b). The endothelial marker gene *cdh5* was

169   expressed in all the clusters (Figure 4c). The notch ligand *dlc* was highly expressed in

170   arterial, capillary ECs, and arch ECs (Figure 4d). The *dab2* and *prox1* were mainly enriched

171   in venous and lymphatic ECs (Figure 4e-f). The *cdk1*, which is a key player in cell cycle

172   regulation, was specifically expressed in proliferating ECs (Figure 4h). It was revealed that

173   the ratio of arterial and capillary ECs and proliferating ECs was increased in the high

174   glucose-treated embryos (Figure 4i, j), consistent with the observation that glucose treatment

175   resulted in excessive sprouting angiogenesis of ISVs.

176   **Foxo1a was significantly downregulated in arterial and capillary ECs**

177   To identify the potential molecules responsible for increasing the proportion of arterial and

178   capillary ECs in the embryos treated with glucose, we analyzed and compared the

179   differentially expressed genes (DEGs) in arterial and capillary ECs of control and glucose-

180   treated ECs. The results revealed that 1201 genes were up-regulated and 523 genes were

181   down-regulated significantly (Figure 5a). GO analysis revealed that these DEGs were

182   enriched in several biological processes, including regulation of actin filament organization,

183   blood vessel morphogenesis, development, angiogenesis, etc. (Figure 5b).

184   Subsequently, we searched for transcription factors among the genes involved in the

185   aforementioned biological processes that might participate in inducing excessive

186   angiogenesis. It has been reported that the loss of function of *foxo1a* led to excessive

187   angiogenesis [34, 35]. Our study also revealed that *foxo1a* was significantly downregulated in

188   arterial and capillary ECs after high glucose treatment compared to the ECs marker gene

189   *pecam1*(Figure 5c-e). The *in situ* hybridization (ISH) experiments further confirmed the

190   decrease in *foxo1a* expression following treatment with high D-glucose and L-glucose

191   (Figure 5f). To verify whether the downregulation of Foxo1a led to excessive angiogenesis in

192   zebrafish embryos, we performed loss-of-function experiments targeting *foxo1a*. AS1842856,

193   a cell-permeable inhibitor that has been reported to block *foxo1* transcription activity [36],

194   was administered to zebrafish embryos at 48 hpf and the imaging was performed at 72 hpf.

195   The results revealed significantly excessive angiogenesis in AS1842856 treated embryos

196   compared with the control group, consistent with the results obtained from *foxo1a* MO

197   injection (Figure 5g-i).

198   **Monosaccharides induced excessive angiogenesis through the *foxo1a-marcksl1a***

199   **pathway.**

200   A previous study has reported that *marcksl1a* overexpression in ECs in zebrafish led to a

201   significant increase in filopodia formation, similar to the phenotype we observed in response

202   to high glucose treatment [37]. Our analysis of the single-cell sequencing data revealed a

203   significant upregulation of *marcksl1a* in arterial and capillary ECs following high glucose

204   treatment, compared to the ECs marker gene *kdrl* (Figure 6a, b). The real-time qPCR and ISH

205   experiments further confirmed the elevated expression levels of *marccksl1a* following high

206   D-glucose and L-glucose treatment (Figure 6c, d). Then, by constructing the transgenic

207   zebrafish line *hsp70l:marcksl1a-p2A-mCherry:: Tg(fli1a:EGFP-CAAX)* [ntu666], we conducted

208   the experiments to overexpress *marccksl1a* in zebrafish and subsequently observed the

209   vascular developmental phenotype. After one hour of heat shock for 1 hour at 24 hpf and

210   confocal imaging analysis at 72 hpf, significantly increased blood vessel formation was

211   observed in embryos overexpressing marccksl1a, compared with the control group (Figure

212   6e-g).

213   Given the results obtained from *marcksl1a* overexpression and loss of function of *foxo1a*,

214   we hypothesized that *marcksl1a* might be a target gene of Foxo1a. Therefore, we investigated

215   the impact of Foxo1 inhibition on marcksl1a expression in zebrafish embryos. As expected,

216   qPCR analysis revealed that inhibition of Foxo1 by AS1842856 resulted in upregulation of

217   *marcksl1a* expression (Figure 7a, b), which suggested that Foxo1a might negatively regulate

218   *marcksl1a* transcription in zebrafish. To further confirm it, we performed the Chromatin

219   Immunoprecipitation (ChIP) experiment to validate the potential binding interaction between

220   Foxo1 and *marcksl1a*. Since the amino acid sequence and DNA binding motifs of Foxo1 are

221   highly conserved between zebrafish and mice (Supplementary Figure 11), we analyzed the 3

222   kb promoter region of *marcksl1a* to search the binding site sequence of mouse FOXO1

223   presented in the JASPAR database. Two candidate binding sites (BS) were found at −265 to

224   −275 (BS1) and −153 to −163 (BS2) nucleotides upstream of the TSS of *marcksl1a* (Figure

225   7c) and then used for the ChIP-PCR assay detection. The results showed that Foxo1a was

226   enriched in both the predicted binding sites of *marcksl1a* (Figure 7d) in zebrafish.

227   Additionally, we microinjected *marcksl1a* MO into the 1-cell stage *Tg(fli1a:EGFP-CAAX)*

228   ^ntu666 embryos, which were then treated with high levels of D-glucose and L-glucose. The

229   findings revealed that the knockdown of Marcksl1a could effectively mitigate the excessive

230   angiogenesis caused by high D-glucose or high L-glucose treatment, resembling the rescue

231   effect observed with VEGFR inhibitor lenvatinib (Figure 7e-k, Supplementary Figure 11).

232   These results suggested that monosaccharides induced excessive angiogenesis through the

233   Foxo1a-*marcksl1a* pathway in zebrafish embryos.

234

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 Jörgens 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. To gain more insights into it, we performed the analysis of single-cell transcriptomic sequencing data of the endothelial cells isolated from D-glucose-treated embryos. 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.

256   Foxo1 has been validated to be essential for sustaining the quiescence of endothelial cells,

257   with involvement in metabolism regulation [34, 40]. Moreover, it also plays important roles

258   in diabetic microvascular complications including DR[41, 42]. Here, by combining the

259   single-cell transcriptomic sequencing data analysis and experimental validation, we identified

260   the transcription factor Foxo1a, which was significantly down-regulated in the embryos

261   treated with high glucose, responsible for the excessive angiogenesis. Additionally, our result

262   further revealed that Foxo1a exerts its regulatory function during this process by down-

263   regulating its target gene *marcksl1a*, regardless of whether the embryos were treated with D-

264   glucose or L-glucose. Taken together, our results suggested that both caloric and noncaloric

265   monosaccharides treatment could lead to excessive angiogenesis by promoting the

266   differentiation of quiescent endothelial cells into tip cells through the *foxo1a-marcksl1a*

267   pathway.

268   The consumption of ASB has been linked to the occurrence and development of

269   cardiovascular disease in previous studies [12, 16-23]. However, the potential mechanisms

270   underlying the association have not been well documented. In recent years, positive

271   associations between ASB and cardiovascular disease have been proposed, possibly due to

272   several plausible factors, including the potential impact of ASBs on central nervous system

273   circuits, gut hormone secretion, and gut microbiota [43-45]. Additionally, it has been

274   hypothesized that the ASBs might stimulate appetite and increase calorie intake [43, 44].

275   For a long time, there has been considerable debate and conflicting opinions regarding how

276   specific sugars affect the development of type 2 diabetes rather than excess calories per se

277   [46, 47]. In this study, we have provided new evidence indicating that the administration of

278   noncaloric monosaccharides leads to significant excessive angiogenesis, suggesting that the

279   excessive angiogenesis may not be only attributed to the caloric properties. Since excessive

280   angiogenesis is the major pathological feature of diabetic retinopathy and nephropathy, our

281   findings are in support of a possible biological mechanism underlying the positive

282   associations between noncaloric monosaccharides and microvascular complications

283   associated with type 2 diabetes, suggesting that the noncaloric monosaccharides might not be

284   suitable for ASB consumption.

285   Surprisingly, no notable abnormalities were observed in the vessels of embryos treated

286   with disaccharides, including lactose, maltose, and sucrose, which is consistent with the

287   previous study stating that intakes of sucrose, lactose, and maltose were not significantly

288   associated with the risk of type 2 diabetes [48]. This finding implied that the effects induced

289   by monosaccharides cannot be attributed to the osmotic pressure of the surrounding medium.

290   Furthermore, despite the potential conversion of these disaccharides into monosaccharides,

291   the restricted reaction rate may maintain them within a safe concentration range that is not

292   harmful to the vessels in a short period.

293   In conclusion, to investigate the effects of monosaccharides on vascular development, we

294   established a zebrafish model by treating the embryos with high concentrations of

295   monosaccharides. Based on this model, we observed significant excessive angiogenesis

296   induced by glucose and noncaloric monosaccharides, initiated by activating the quiescent

297   endothelial cells into proliferating tip cells. The effects of monosaccharides on inducing

298 excessive angiogenesis were then proved to be mediated by the *foxo1a-marcksl1a* pathway.

299 The results have provided novel insights into the roles of noncaloric monosaccharides in

300 human health and the underlying mechanisms.

301 **Materials and methods**

302 **Zebrafish**

303 Care and breeding of zebrafish were carried out as previously described [49]. Animal

304 experiments were conducted according to local institutional laws and Chinese law for the

305 Protection of Animals. The following transgenic strains were used: *Tg(fli1aEP:EGFP-*

306 *CAAX)^{ntu666}* and *Tg(kdrl:ras-mCHerry* [33]. Embryos were obtained through natural mating

307 and maintained at 28.5°C. The stages of zebrafish embryos are defined as previously

308 described [49]. Embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma,

309 P7629) to block pigmentation for further imaging analysis.

310 **Monosaccharides and drug treatment**

311 The D-glucose (Sigma, G7021-100g), L-glucose (Sigma, G5500-1g; J&K, 981195-1g), D-

312 Fructose (Sigma, F0127-100g), L-Rhamnose monohydrate (Aladdin, R108982), D-Sorbitol

313 (Sigma, S1876-100g), D-Mannitol (Sigma, M4125-100g), D-(-)-Ribose (Sigma, V900389-

314 25g), L-(+)-Arabinos (Sigma, V900920-25g), Mannose (Sigma, M2069-25g) and sucrose

315 (Sigma, V900116-500G) were dissolved in E3 solution. Zebrafish embryos at 24 hpf to 48

316 hpf were placed in 24-well plates (ten embryos per well) and immersed in the solution at the

317 presetting concentrations and time windows. Then, put it in a 28 °C incubator for cultivation.

318 Five days before embryonic development, a stereo fluorescence microscope and a laser

319   confocal microscope were used to observe the changes in blood vessel phenotype. For the

320   drug treatment, the embryos were co-incubated in glucose with lenvatinib (Selleck, S1164-

321   5MG) from 48 hpf to 96 hpf. Foxo1 inhibitor AS1842856 (MCE, HY-100596) was dissolved

322   in DMSO and stored at -80℃ and diluted with E3 solution when used. The same

323   concentration of DMSO was used as a negative control.

324   **Glucose concentration measurement**

325   Glucose concentration in the embryo was measured as described previously [50]. Embryos

326   that developed to 75% epiboly were selected and transferred to 24-well plates (ten embryos

327   per well) and immersed in the solution at the presetting concentrations and time windows.

328   For glucose concentration measurement, embryos (n=20) were transferred to a new 1.5 mL

329   tube, rinsed three times with 1×PBS, and immersed in ice for the following experiments.

330   Discard the PBS as much as possible, embryos homogenized using a hand homogenizer, and

331   centrifuged at 14,000×g for 10 min. 1.5 μL of the supernatant was used to measure the total

332   free-glucose level using a glucometer (Baye, 7600P).

333   **Whole-mount in situ hybridization (WISH)**

334   Whole-mount in situ hybridization and the preparation of antisense RNA probes were

335   performed as described in the previous protocol [51]. Briefly, the *marcksl1a* and *foxo1a*

336   cDNA fragments were cloned with the specific primers listed below using the wild-type

337   embryo (AB) cDNA library. Probes were synthesized using the in vitro DIG-RNA labeling

338   transcription Kit (Roche, 11175025910) with linearized pGEM-T easy vector containing

339   *marcksl1a* or *foxo1a* gene fragment as the templates. Synthesized probes were purified with

340   LiCl (Invitrogen, AM9480) and diluted to 1 ng/µL for hybridization. Zebrafish embryos were

341   collected and fixed with 4% paraformaldehyde (PFA) overnight at 4˚C, then dehydrated with

342   methanol gradients and stored at -20℃ in 100% methanol. The hybridization result was

343   detected with anti-DIG-AP antibody (1:2000, Roche, 11093274910) and NBT/BCIP (1:500,

344   Roche, 11681451001). After hybridization, images of the embryos were captured with an

345   Olympus stereomicroscope MVX10. The primers are listed below:

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

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

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

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

350   **Single-cell gene expression profile analysis**

351   Cell Ranger 3.0.2 (https://github.com/10XGenomics/cellranger) was used to convert the

352   raw sequencing data to a single-cell level gene count matrix. The clustering of single cells

353   and the marker genes in each cluster were analyzed by Seurat 3.0

354   (https://satijalab.org/seurat/install.html) [52]. Several criteria were applied to select the single

355   cells, including only keeping the genes that are expressed (Unique Molecular Identifiers or

356   UMI larger than 0) at least in 3 single cells, selecting single cells with the number of

357   expressed genes at the range between 500 and 3000, and requiring the percentage of

358   sequencing reads on mitochondrial genome being less than 5 percentage. Furthermore,

359   sctransform method [53] was applied to remove technical variation, and ClusterProfiler [54]

360   was used to do the Gene Ontology enrichment analysis based on the marker genes of each

361   cell cluster. Detailed information about the data processing can be found in the source code of

362   this project (https://github.com/gangcai/ZebEndoimmune).

363   **Gene expression analysis by quantitative real-time PCR**

364   Total RNA was extracted from zebrafish embryos using TRIzol™ (Invitrogen, 15596026),

365   and stored at -80℃. The cDNA was then synthesized using the HiScript II Q RT SuperMix

366   for qPCR Kit (Vazyme, R223-01) according to the manufacturer's instructions. Quantitative

367   PCR was performed in triplicates using the Taq Pro Universal SYBR qPCR Master Mix

368   (Vazyme, Q712-02) on a real-time PCR detection system (StepOne™ Real-Time PCR

369   Systems). The primers used for Real-time PCR analysis are as follows:

370   ef1α-Qpcr-F:5'- CTT CAA CGC TCA GGT CAT CA -3'

371   ef1α-Qpcr-R:5'- CGG TCG ATC TTC TCC TTG AG -3

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

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

374   **Transgenic and heat shock**

375   The *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* line was established using a construct *fli1aEP:EGFP-*

376   *CAAX*, which was generated using multisite Gateway technology, the tol2 kit as previously

377   described [55]. The 5' Entry p5Efli1ep (#31160) purchased from Addgene was originally

378   from Nathan Lawson Lab [56]. Three entry clones and the pDestTol2pA2 destination vector

379   were used to generate the expression construct by LR recombination reaction as described in

380   the Multisite Gateway Manual book. The expression constructs were synthesized by

381   GENEWIZ company. The zebrafish embryos were immersed in a 37 ℃ water bath for 1 hour

382   for heat shock. Around 75 pg of expression plasmid DNA and 25 pg tol2 transposase mRNA

383   were premixed and microinjected into single-cell fertilized eggs.

Chip-PCR

385   Embryos injected with *hsp70l:foxo1a-6×His-P2A-mCherry* were collected at 72 hpf after

386   heat shock treatment. According to the manufacturer's instructions, the ChIP-PCR assay was

387   performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore, 3753379).

388   The genomic DNA crossed with Foxo1a protein was immunoprecipitated by using 5 μg Anti-

389   6×His tag antibody (abcam, ab213204). Antibody against lgG was used as a negative control.

390   The semiquantitative PCR was performed with KODfx (TOYOBO, KFX-101) at the

391   following conditions: 94°C for 5 min; 35 cycles of 98°C for 10 s, 55°C for 30 s, 68°C for 10

392   s; 68°C for 10 min. The PCR primers used for the predicted binding sites (BS) are as follows:

393   Marcksl1a-BS1-forward:5'- CCC TTT TTC AAA AGT GAG TTT GAG -3'

394   Marcksl1a -BS1-reverse:5'- GGA GCT TCA TCT GCC CCA TT -3'

395   Marcksl1a -BS2-forward:5'- CGG TTT CCA GCT TTC TTC AGA A -3'

396   Marcksl1a -BS2-reverse:5'- TCT CAA ACT CAC TTT TGA AAA AGG G -3'

Imaging analysis

398   For confocal imaging of blood vessels in fluorescence protein labeled transgenic zebrafish

399   embryos, they were anesthetized with egg water/0.16 mg/mL MS222 (Sigma, A5040)/1%

400   PTU and embedded in 0.5-0.8% low melting agarose. Confocal imaging was performed with

401   a Nikon A1R HD25 Confocal Microscope. Analysis was performed using Nikon-NIS-

402   Elements software. The bright field images were acquired with an Olympus DP71 camera on

403   an Olympus stereomicroscope MVX10.

Statistical analysis

405   Statistical analysis was performed with a student's t-test. All data is presented as Mean ±

406   SEM; $p < 0.05$ was considered statistically significant.

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Conflicts of interest/Competing interests

411   The authors declare that they have no conflicts of interest

Availability of data and material (data transparency)

413   All the experimental materials generated in this study are available from the corresponding

414   authors upon reasonable request.

Authors' contributions

416   Dong Liu, Xuchu Duan, Xia Liu, and Gangcai Xie conceived and designed the

417   experiments and wrote the manuscript. Xiaoning wang, Jinxiang Zhao, Jiehuan Xu, Bowen

418   Li, and Gangcai Xie performed the experiments and analyzed the data. All authors read and

419   approved the final manuscript.

Ethics approval

421   All zebrafish experimentation was carried out following the NIH Guidelines for the care

422   and use of laboratory animals (http://oacu.od.nih.gov/regs/index.htm) and ethically approved

423   by the Administration Committee of Experimental Animals, Jiangsu Province, China

424   (Approval ID: 20180905-002).

425   **Acknowledgments**

426   Not applicable

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576

577

Figure Legends

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

600   control and glucose-treated embryos. t-test, ****p<0.0001. **o**, A diagram showing the blood

601   vessels in position 2 indicated in panel b of control embryos. **p**, A diagram showing the blood

602   vessels in position 2 indicated in panel b of high glucose-treated embryos.

603

604   **Figure 2 L-glucose and mannose treatment caused excessive angiogenesis as well. a,** A

605   diagram showing the monosaccharides treatment time window and imaging time point. **b**, A

606   diagram indicating the imaging position of the zebrafish embryos. **c-g**, Confocal imaging

607   analysis of the control and monosaccharides, including L-glucose, D-mannose, D-ribose, and

608   L-arabinose, treated embryos. Arrowheads indicate the ectopic branching from the dorsal

609   aorta. Stars indicate the ectopic vessels from ISVs. **h**, Statistical analysis of the total length of

610   ISVs in control and monosaccharides treated embryos. t-test, ****p<0.0001.

611

612   **Figure 3 High glucose treatment induced endothelial differentiation into tip cell-like**

613   **cells. a,** A diagram showing the confocal time-lapse imaging time window. **b**, A diagram

614   indicating the imaging position of the zebrafish embryos. c, Confocal time-lapse imaging

615   analysis of blood vessels in control *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **d**, A diagram

616   showing the glucose treatment time window and confocal time-lapse imaging time window.

617   **e**, Confocal time-lapse imaging analysis of blood vessels in glucose-treated

618   *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Arrowheads indicate the ectopic angiogenic

619   branches. **f**, A snapshot of confocal time-lapse imaging analysis of blood vessels in glucose-

620   treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Z stacks were used to make 3D color

621   projections, where blue represents the most proximal (closest to the viewer), and red

622   represents the most distal (farthest from the viewer). Arrowheads indicate ectopic angiogenic

623   sprouts. **g,** A snapshot of confocal time-lapse imaging analysis of an ISV in glucose-treated

624   *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. Arrowheads indicate ectopic angiogenic sprouts.

625

626   **Figure 4 Single-cell transcriptome sequencing analysis of endothelial cells in control and**

627   **high glucose treated embryos. a**, Schematic diagram of the single-cell sequencing process.

628   300 embryos in the control group and 300 embryos in the high glucose group were used, and

629   ECs were sorted by GFP fluorescent using FACS technology. **b**, The measured cells were

630   divided into 6 individual clusters based on gene expression profiles using UMAP. **c-h**, The

631   violin plots of some endothelial cell marker genes. **i**, The proportion of ECs in each cluster of

632   the control and high glucose groups. **j**, Changes of ECs percentage in arterial and capillary

633   ECs, endocardium, and proliferating ECs of control and high glucose group.

634

635   **Figure 5 Foxo1a was involved in the excessive angiogenesis induced by high glucose**

636   **treatment. a**, The volcano plot of differential expression genes in arterial and capillary ECs.

637   The avg_log2FC greater than 1 was considered significant, including 523 down-regulated

638   genes (blue dots) and 1201 up-regulated genes (red dots). **b**, GO analysis of 523 down-

639   regulated genes in arterial and capillary ECs. **c,** The feature plot of ECs marker gene pecam1

640   of control and high glucose group in arterial and capillary ECs. **c',** The violin plot of ECs

641   marker gene pecam1 of control and high glucose group in arterial and capillary ECs. **d**, The

642   feature plot of gene foxo1a of control and high glucose group in arterial and capillary ECs.

643   **d',** The violin plot of gene foxo1a of control and high glucose group in arterial and capillary

644   ECs. **e**, Average expression of gene pecam1 and foxo1a in control and high glucose group. **f**,

645   Whole-mount in situ hybridization analysis of foxo1a in control, high glucose, and high L-

646   glucose treated embryos. **g**, A diagram showing the foxo1 inhibitor treatment time window. **h**,

647   Confocal imaging analysis of control embryos, AS1842856 treated embryos, and foxo1a MO-

648   injected embryos. **i**, Statistical analysis of the total length of ISVs in control embryos,

649   AS1842856 treated embryos, and foxo1a MO-injected embryos. *t-test*, ****p<0.0001.

650

651   **Figure 6 Marcksl1a over-expression induced excessive angiogenesis in zebrafish**

652   **embryos. a**, The violin plot of ECs marker gene kdrl of control and high glucose group in

653   arterial and capillary ECs. **b**, The violin plot of gene marcksl1a of control and high glucose

654   group in arterial and capillary ECs. **c,** Real-rime PCR analysis of marcksla1a expression in

655   control, high glucose, and high L-glucose treated embryos. *t-test*, ****p<0.0001. **d**, Whole-

656   mount in situ hybridization analysis of mmarcksl1a in control, high glucose, and high L-

657   glucose treated embryos. **e-f'**, Confocal imaging analysis of blood vessels in control and

658   *hsp70l:marcksl1a-P2A-mCherry* injected *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **g**,

659   Statistical analysis of the total length of ISVs in control and *hsp70l:marcksl1a-P2A-mCherry*

660   injected embryos. *t-test*, **p<0.01.

661

662   **Figure 7 Noncaloric monosaccharides induced excessive angiogenesis through foxo1a-**

663   **marcksl1a signal in zebrafish embryos. a**, A diagram showing the Foxo1 inhibitor

664   treatment time window. **b**, Real-rime PCR analysis of marcksla1a expression in control and

665   AS1842856 treated embryos. Two-way ANOVA, **p<0.01, ***p<0.001*. **c**, A sequence logo

666   of Foxo1 binding sequence presented in JASPAR database (https://jaspar.genereg.net/) and

667   two candidate binding sites at the upstream of transcription start site (TSS) of mmarcksl1a in

668   zebrafish. **d**, Results of the ChIP-PCR assay indicated that BS1 and BS2 are Foxo1a-binding

669   sites of marcksl1a in zebrafish. Input sonicated genomic DNA samples without

670   immunoprecipitation as a positive control. IgG, sonicated, and IgG-immunoprecipitated

671   genomic DNA samples as a negative control. **e-j**, Confocal imaging analysis of blood vessels

672   in control, high glucose, high glucose & Lenvatinib, high glucose+marcksl1a MO, high L-

673   glucose and high L-glucose+marcksl1a MO groups. **k**, Statistical analysis of the total length

674   of ISVs in the groups in figure e-j, respectively. one-way ANOVA, ****p<0.0001.

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Supplementary Figure Legends

Supplementary Figure 1 Confocal imaging analysis of *Tg(fli1aEP:EGFP-CAAX)^{ntu666}*
embryos at 48 hpf (a) and 3 dpf (b).

Supplementary Figure 2 The diagrams show the glucose treatment time window and
imaging time point.

Supplementary Figure 3 Total glucose concentrations at different development stages in
control and high glucose treated embryos. **a**, A diagram showing the glucose treatment time window and concentration measuring time point. **b**, Statistical analysis of the glucose concentration in control and high glucose-treated embryos. one-way ANOVA, ****$p<0.0001$.

Supplementary Figure 4 Stereo microscopic analysis of control, glucose, and sucrase-
treated embryos in a bright field. a-c, Imaging analysis of control, glucose, and sucrase treated embryos in bright field. **d**, A diagram showing the sucrase treatment time window and imaging time point. **e**, A diagram indicating the imaging positions of the zebrafish embryos. **f**, Confocal imaging analysis of the control and sucrase treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. The red bar indicates position 1; the green bar indicates the position. **g**, A diagram showing the sucrase treatment time window and imaging time point. **h**, Confocal imaging analysis of the control and sucrase treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. The red bar indicates position 1; the green bar indicates the position.

702

703 **Supplementary Figure 5 Confocal imaging analysis of 1% - 4% glucose treated blood**

704 **vessels. a**, A diagram showing the glucose treatment time window and imaging time point. **b-**

705 **g'**, Confocal imaging analysis of the control, 1%, 2%, 3%, and 4% glucose-treated

706 *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos.

707

708 **Supplementary Figure 6 Fructose treatment caused excessive angiogenesis in zebrafish.**

709 **a**, A diagram showing the fructose treatment time window and imaging time point. **b**, A

710 diagram indicating the imaging position of the zebrafish embryos. **c-d**, Confocal imaging

711 analysis of the control and glucose-treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. e-f,

712 Imaging analysis of control and fructose treated embryos in bright field.

713

714 **Supplementary Figure 7 Lactose and maltose treatment did not cause excessive**

715 **angiogenesis in zebrafish. a**, A diagram showing the lactose and maltose treatment time

716 window and imaging time point. **b-d"**, Confocal imaging analysis of the control, lactose, and

717 maltose treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos.

718

719 **Supplementary Figure 8 Pyruvic acid treatment did not cause excessive angiogenesis in**

720 **zebrafish. a**, A diagram showing the pyruvic acid treatment time window and imaging time

721 point. **b**, Confocal imaging analysis of the pyruvic acid-treated *Tg(fli1aEP:EGFP-*

722 *CAAX)^{ntu666}* embryos. **c**, A diagram showing the pyruvic acid treatment time window and

723 imaging time point. **d**, Confocal imaging analysis of the pyruvic acid-treated

724   *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos.

725

726   **Supplementary Figure 9 Overview of the number of genes, total UMIs, and percentage**

727   **of mitochondrial UMIs for the single-cell RNA sequencing. a**, Before filtering. **b**, After

728   filtering. Cell selection criteria: 500< number of genes < 3000; 0< percentage of

729   mitochondrial UMIs < 5%.

730

731   **Supplementary Figure 10 UMAP representation of EC subpopulations. All single cells**

732   **(after filtering) from the control and D-glucose treated were included in this illustration.**

733

734   **Supplementary Figure 11 Confocal imaging analysis of blood vessels in the embryos**

735   **with lenvatinib treatment. a**, A diagram showing the lenvatinib treatment time window and

736   imaging time point. **b**, Confocal imaging analysis of the control and lenvatinib treated

737   *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos. **c**, A diagram showing the lenvatinib treatment time

738   window and imaging time point. **d**, Confocal imaging analysis of the control and lenvatinib

739   treated *Tg(fli1aEP:EGFP-CAAX)^{ntu666}* embryos.

a kdrl

b marcksl1a

c

d ctrl Glucose L-Glucsoe

Tg(fli1ep:EGFP-CAAX) 72 hpf

e Ctrl

f +marcksl1a *Tg(hsp70l:marcksl1a-P2A-mCherry)* HS@24 hpf

f' +marcksl1a

g