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C1qr and C1qrl redundantly regulate angiogenesis in zebrafish through controlling endothelial Cdh5

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ABSTRACT

Angiogenesis plays central role in the formation of functional circulation system. Characterizations of the involved factors and signaling pathways remain to be the key interest in the angiogenesis research. In this report, we showed that *c1qr*/*cd93* and *c1qrl*/*clec14a* are specifically expressed in the vascular endothelial cells during zebrafish development. Single mutation of *c1qr* or *c1qrl* is associated with slightly malformation of inter-segmental vessels (ISVs), whereas double mutant exhibits severe defects in the ISVs formation without affecting early vasculogenesis. Further studies reveal that the endothelial-endothelial junctional molecule Cdh5 becomes absent in the ISVs of the double mutant. Replenishment of Cdh5 efficiently rescue the impaired angiogenesis in the *c1qr*/*c1qrl* double mutant. These data demonstrate that *c1qr* and *c1qrl* redundantly regulate angiogenesis through controlling the expression of the endothelial junctional molecule Cdh5, thus playing an important role in angiogenesis.

1. Introduction

The vascular network supplies all organs with oxygen and nutrients, and plays an important role during embryonic development and physiologic processes [1]. The embryonic vascular network morphogenesis of the vertebrate involves two phases (vasculogenesis and angiogenesis) [2–4]. Vasculogenesis is a process of differentiation and coalescence of angioblasts from the lateral mesoderm [3]. The complex process of angiogenesis, including endothelial cell proliferation, tip/stalk cell formation, migration, extracellular matrix remodeling, and lumen formation, is managed by angiogenic signaling networks [4]. Numerous papers have reported pro- and anti-angiogenic factors participating in angiogenesis, among which Vegf is an important mediator that promotes angiogenesis. Vegf exhibits its biologic function by binding to and activating its main receptor, Vegfr2 [5,6]. With the exception of Vegf-Vegfr2 signaling, a number of pathways are involved in angiogenesis, including Dll4-Notch1a [7], Wnt [8], and the cell adhesion pathway. The correct pattern and integration of vascular endothelial cell–cell adhesion drives the morphogenesis of new vessels [9]. Vascular endothelial–cadherin (Cdh5) is known as the major cadherin that connects all endothelial cells [10]. Vascular endothelial cells also express and release protein components of the complement cascade [11], but the relationship between the complement system and endothelial cell formation is still unclear.

Both C1qr and C1qrl are members of the type 14 family of calcium-dependent C-type lectins and have a role in tumor formation and immunity [12]. A component of the complement system, C1qr, is a heavily glycosylated transmembrane protein that is preferentially expressed in the vascular endothelium. A previous report demonstrated that silencing *c1qr* impairs endothelial cell proliferation, migration and sprouting by shRNA expression targeting *c1qr* in HUVECs [13]. A transmembrane protein with C-type lectin and EGF domains, C1qrl, is a novel tumor endothelial marker because of high expression in tumor vessels compared to the vasculature in healthy tissues [12]. Although C1qr and C1qrl likely influence vascular endothelial cell behavior in HUVECs or tumor vessels *ex vivo*, there is still a lack of evidence to support that C1qr

Abbreviations: hpf, hours post fertilization; Vegfa, vascular endothelial growth factor A; ISVs, inter-segmental vessels; DLAVs, dorsal longitudinal anastomotic vessels; Cdh5, vascular endothelial cadherin 5.

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and C1qrl are also necessary for vasculogenesis or angiogenesis *in vivo*.

Recent advances with the type II clustered regularly interspaced short palindromic repeats (CRISPR) system promise an improved approach to genome editing. The CRISPR-Cas9 system has been successfully applied in a variety of mammalian cells and zebrafish [14]. Indeed, the function of specific genes by the knockout system of CRISPR-Cas9 has attracted increased research interest. It has been suggested that there is no phenotype by this system because of complex regulation of protein levels [15]; however, this complex regulation is not suitable for all genes in an organism.

Zebrafish serve as a powerful model animal to study vertebrate organ development, such as the vasculature and liver [16–19]. Many of the genes identified are also important elements in vascular pathologies through traditional forward-genetic screens for mutants with vascular phenotypes [17]. Recently, there has been a preference to understand specific gene function with development of genome-engineering technologies. We obtained two frame-shift mutants of *c1qr* and *c1qrl* using the knockout system of CRISPR-Cas9. Surprisingly, we found that the double mutant has a more serious phenotype of blood vessels than single mutant and cell adhesion signaling may play a role in this process. These data can help us understand the mechanisms of angiogenesis in zebrafish.

2. Materials and methods

2.1. Zebrafish strains

Zebrafish (*Daniorerio*) of the AB genetic background and *Tg(flk1:GFP)* were maintained under standard laboratory conditions according to institutional animal care and use committee protocols.

2.2. In Situ Hybridization

Whole mount in situ hybridization was performed as previously described [18,19] using the *c1qr*, *c1qrl*, and *etv2*, *cdh5*, *vegfa* probes. Primer Sequences, *c1qr*-FP: GCGCGTCTTCAGCTCTAAAC, *c1qr*-RP: GAGTTCAGTGCACTCGTCCAC; *c1qrl*-FP: GGAGAAAAAGCAGACAATATCATTTTA, *c1qrl*-RP: AGTCTCTCTCACTTAGGTTTCCTCTTT.

2.3. Microinjection of synthetic mRNAs

Total RNA was extracted using Trizol (Life Technologies) and reverse transcribed to cDNA using Omniscript reverse transcriptase kit (QIAGEN). Zebrafish *c1qr, cdh5* and *c1qrl* CDS fragment were amplificated and sub-cloned into the PCS2+, the mRNA expression vector. Capped mRNAs were synthesized by using the mMESSAGE (Ambion). Synthetic mRNAs were injected into the blastomere of 1- to 2- cell-stage embryos.

2.4. Establishing mutant lines

CRISPR/Cas9 mutations were generated in *Tg(flk1:GFP)* (GO strain) zebrafish using published techniques [14,20]. Fertilized one cell stage zebrafish eggs were injected with an injection mix containing approximately 300 ng/μl Cas9 mRNA and 25 ng/μl sgRNA. Confirm the definite mutation and obtain stable lines through a series of PCR, cloning and sequencing.

2.5. Genotyping

For genotyping, we extracted genomic DNA from whole larvae using the 50 mM NaOH and neutralized with Tris-Hcl. We amplified *c1qr* and *c1qrl* target DNA using following primers, *c1qr*-FP: GCACGGCTTCAGGTGGACTTC, *c1qr*-RP: CGCAGTTGTCTTTGGCAGCAC. These primers amplify a 538-bp region including the *c1qr* mutation site. *c1qrl*-FP: CGGCTGCTTCATTTTGGGTTGG, *c1qrl*-RP: GGTATCTCCGGTCGTTGGCAC. These primers amplify a 291-bp region including the *c1qrl* mutation site.

2.6. Quantitative real time PCR

Quantitative real-time PCR was performed as previously described [19]. Primer Sequences, *cdh5*-FP: GACGAGATACAAAACAAAGATC, *cdh5*-RP: CACAATTACGATAAACTTGTG; *c1qr*-FP: GAACCGCTCACTCTTGATGAAC, *c1qr*-RP: CCGGTGCGCAAAAGCGCTCAG; and *c1qrl*-FP: GGATTCCACTGGACGGTGGAC, *c1qrl*-RP: GGATGTTGCTGTTTGCAGGTG. Transcription of *b-actin*-FP: CCACGAGACCACTTTCAACTC, *b-actin*-RP: GTGCTAGGGGCCAGGGATGTG was used for normalization.

2.7. Heartbeat rate and survival measurements

After 72 hpf, wild type and mutant larvae were taken a 30 s videos using using ZEN2010 software equipped on an LSM780 confocal microscope (Carl Zeiss). Then the beating heart was outlined and heart rate was calculated.

Larval zebrafish were maintained as described above and counted daily. They were collected and genotyped when dying. Data were plotted using Microsoft Excel.

2.8. Microscopy

Whole mount in situ hybridized larvae were imaged using aSteREODiscoveryV20 microscope equipped with AxioVisionRel 4.8.2 software (Carl Zeiss, Jena, Germany). Antibody stainedand live larvae were imaged using ZEN2010 software equippedon an LSM780 confocal microscope (Carl Zeiss).

3. Results

3.1. c1qr and c1qrl are specifically expressed in zebrafish endothelial cells

The first sub-component of the C1 complex of the classical pathway of complement activation is C1q. Both C1qr and C1qrl are complement receptors, but these two receptors appear to have less of a role in C1q function [21]. It has been shown that *c1qr* and *c1qrl* are specifically expressed in endothelial cells [22] and have a role in migration and sprouting of endothelial cells *in vitro*. However, no evidence proves that these two genes influence vascular network formation *in vivo*. In zebrafish, both genes are located on chromosome 17 and share C-type lectin (CTL) and calcium-binding epidermal growth factor (EGF)–like homology domains. Although these two proteins have similar domains, C1qrl is unlikely to be an ortholog of C1qr [22]. The *c1qrl* cDNA contains a complete ORF encoding a zebrafish protein of 368 AA and *c1qr* RNA encoding a protein of 528 AA. Both two genes are expressed in endothelial cells from 20 h post-fertilization (hpf) to 60 hpf (Fig. 1A, B and E, F). Moreover, *c1qr* and *c1qrl* expression were absent in the *cloche* mutant, suggesting that these genes are expressed in vasculature specifically (Fig. 1C, D and G, H). Therefore, we suspected that C1qr and C1qrl may play a role during vascular formation or morphogenesis in zebrafish.

3.2. Knockout of c1qr and c1qrl by CRISPR-Cas9 in zebrafish

In order to determine whether or not C1qr and C1qrl affect vascular network formation or remodeling in zebrafish, we

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