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Expression of zTOR-associated microRNAs in zebrafish embryo treated with rapamycin

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ABSTRACT

Aims: MicroRNAs (miRNAs) are vital in modulating lifespan and various biological processes including vascular function. The pivotal roles of mammalian target of rapamycin (mTOR) in regulating senescence and angiogenesis have been extensively described. However, the roles of its orthologue, zebrafish target of rapamycin (zTOR) in senescence and angiogenesis remain to be unravelled. In the present study, we aimed to investigate the role of zTOR and identify miRNAs associated with senescence and angiogenesis.
Main methods: Zebrafish embryos were treated with rapamycin and the inhibition of zTOR and its downstream proteins were validated by immunoblotting. Following the treatment, melanocyte density was quantitated, and senescence and angiogenic responses were determined by senescence-associated beta-galactosidase (SA-β-gal) and endogenous alkaline phosphatase (ALP) staining, respectively. Relative expression of microRNAs were determined by quantitative RT-PCR.
Key findings: Rapamycin (400nM) suppressed zTOR pathway by down-regulating the phosphorylation of zTOR-associated proteins such as P70S6K and S6K at both 4 h post-fertilisation (hpf) and 8 hpf while 4E-BP1 was only down-regulated at 8 hpf when compared to their respective vehicle controls. Treatment with rapamycin also resulted in significant suppression of melanocyte development and senescence-associated beta-galactosidase (SA-β-gal) activity, and perturbed the development of intersegmental vessels (ISVs) of zebrafish embryos. In addition, the expressions of dre-miR-9-5p and -3p, dre-miR-25-3p and dre-miR-124-3p were significantly up-regulated in embryos treated with rapamycin from 4 hpf.
Significance: Our findings suggest the involvement of zTOR in embryonic senescence and angiogenesis which could be potentially mediated by selected miRNAs.

1. Introduction

Mammalian/mechanistic target of rapamycin (mTOR) is a conserved giant serine/threonine kinase that plays a crucial role in regulating a wide variety of eukaryotic physiological functions by sensing the integrated signals including nutrients, growth factors, stresses and energy. mTOR regulates cell growth, cell proliferation and cell survival in response to signals through the regulation of translation of several downstream mTOR proteins [1]. It is evident that the TOR signalling pathway plays a crucial role in embryonic development in *Caenorhabditis elegans* and *Drosophila melanogaster* [2–4]. It has also become known that the mTOR pathway is essential for angiogenesis [5,6], with studies having shown that inhibition of mTOR suppresses vascular endothelial growth factor (VEGF) expression, hence mitigating angiogenesis [7,8]. In addition, mTOR has recently emerged as a crucial pathway alongside the insulin/insulin-like growth factor-1 (IGF-1) signalling (ISS) pathway, heat-shock factors (HSFs), AMP-activated protein kinases (AMPKs) and sirtuins in regulating longevity [9]. That inhibition of mTOR prolongs lifespan has been demonstrated in invertebrates, including yeast and nematodes, and even in vertebrates such as mice [10–12], shedding light on the role of mTOR in senescence. Attenuation of mTOR had also decelerated senescence in vitro [13,14] and intriguingly, an in vivo study demonstrated that mitigation of mTOR suppressed brain senescence in rats [15].

Rapamycin, an antibiotic derived from the bacterium *Streptomyces hygroscopicus* acts as the canonical inhibitor for mTOR [16]. Rapamycin specifically inhibits mTOR [17] by interacting with its intracellular receptor protein FKBP12 [18]. Due to its high specificity to mTOR, rapamycin has been widely used to study the role of mTOR in cell biology [17]. zTOR, the zebrafish mTOR is highly conserved and is nearly 90% identical to mammalian orthologues including human and mouse. Makky et al. reported that zTOR regulates the developmental process guiding epithelial morphogenesis in the zebrafish intestine [19]. Besides, rapamycin-treated adult zebrafish demonstrated a long-term cardio-protective effect [20].

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MicroRNAs (miRNAs) have recently emerged as critical regulators in modulating the magnitude of gene expressions in organisms [21]. miRNAs are highly conserved 20–25 nucleotides non-coding RNAs that post-transcriptionally regulate gene expressions by binding to the 3′ untranslated region of targeted mRNA and blocking the translation by decreasing its stability [22]. These new molecular regulators have been reported to be de-regulated in pathological conditions [23,24]. Pro-angiogenesis microRNA, miR-126 has been reported to regulate the development of angiogenesis in mice and zebrafish [25,26]. Meanwhile, miR-34 was found to be up-regulated during aging [27].

The teleost fish, zebrafish (*Danio rerio*) has emerged as a prominent animal model in studying complex biological processes, as well as for modelling angiogenesis and senescence due to its rapid angiogenic sprouting that can be clearly visualised by 72 h post-fertilisation (hpf) [28] and its resemblance of gradual senescence to the humans' [29], respectively. The underlying molecular mechanism of how zTOR regulates senescence and angiogenesis remains poorly understood. The current study was designed to identify miRNAs governing zTOR in modulating senescence and angiogenesis in zebrafish embryos. Elucidation of the mechanisms regulating senescence and angiogenesis will provide new insight into the role of specific miRNAs in regulating age-related vascular pathology.

2. Materials and methods

2.1. Animals and ethics statement

Wild type zebrafish embryos (*Danio rerio*) were obtained from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited Zebrafish Laboratory, Department of Biomedical Science, Faculty of Medicine, University of Malaya. All experimental procedures using zebrafish were performed in accordance with relevant guidelines and regulations approved by the Faculty of Medicine, University of Malaya Institutional Animal Care and Use Committee (IACUC) protocol # 2014-04-01/PHAR/R/WPF.

2.2. Zebrafish husbandry

Wild type zebrafish adult (*Danio rerio*) were maintained and housed in the Zebrafish Laboratory under a 14-h light and 10-h dark cycle in a recirculating system (Tecniplast ZebTEC, Italy). Water parameters including temperature, pH and conductivity were monitored daily. Fish were fed with dry food pellets and live *Artemia salina* three times a day. Embryos were collected following successful spawning by adult fish and incubated at 28 °C in system water containing methylene blue. At 1 day post-fertilisation (dpf), embryos for whole mount staining and imaging were transferred into system 0.003% 1-phenyl 2-thiourea (PTU) in order to inhibit melanisation.

2.3. Rapamycin treatment

Healthy zebrafish embryos were transferred into 96-well plates with each well containing 5 embryos. Embryos were treated at 4 hpf or 8 hpf with a total volume of 250 μL per well at the final concentration of 400 nM rapamycin or 0.04% DMSO as vehicle control. Treatments were performed at 4 hpf or 8 hpf with minor modifications by referring to previous reports on the spatiotemporal expressions of zTOR in embryos [30] and the time-points for effective down-regulation of zTOR [31]. All solutions were refreshed daily up till 72 hpf. Zebrafish embryos treated from 4 hpf (68 h of total exposure time to rapamycin) and 8 hpf (64 h of total exposure time to rapamycin) respectively were collected at 3 days post-fertilisation (dpf) for further examination.

2.4. Protein extraction

Thirty to fifty zebrafish embryos were euthanised at 3 dpf with 10% benzocaine and placed into 1.5 mL micro-centrifuge tubes. Embryos were de-chorionated mechanically and then de-yolked with pre-chilled buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaHCO_3$) at 1 μL per embryo on ice while pipetting embryos up and down with a 200 μL pipette tip. Tubes were centrifuged for 5 min at 1100 rpm at room temperature, after which supernatant was removed. Next, every 1 g of embryo pellet was re-suspended with a 20 mL mixture of pre-chilled tissue protein extraction reagent (T-PER; Thermo Scientific) and halt protease & phosphate inhibitor cocktail, EDTA free (100×) (Thermo Scientific) in a 100:1 ratio prior to homogenisation on ice with ultrasonic homogenizer. After that, lysed tissue was incubated on ice for 30 min prior to centrifugation at 15,000 ×*g* for 15 min at 4 °C.

2.5. Total RNA and miRNAs isolation

Thirty to fifty vehicle control and rapamycin-treated zebrafish embryo lysates were homogenised and total RNA was extracted using miRNeasy mini kit (Qiagen) according to the manufacturer's instruction. Purity and integrity of total RNA were assessed using NanoDrop 2000 (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies).

2.6. Protein expression analysis by western blotting

Vehicle control and rapamycin-treated zebrafish embryo lysates were prepared in T-PER buffer. Protein was quantified using bicinchoninic acid protein assay (BCA; Thermo Scientific). Protein lysates (20 μg) were loaded into each lane on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. After running at 100 V, the proteins were transferred to an immobilon-P polyvinylidene difluoride membrane (PVDF; Milipore) at 110 V. The PVDF membranes were incubated in blocking solution with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for about 1 h in room temperature with constant shaking prior to overnight incubation with the respective primary antibody at 4 °C. The primary antibodies included p-P70S6K (thr389) (Cell Signalling, #9205), P70S6K (BD Biosciences, #611,261), p-rpS6 (Ser235/236) (Cell Signalling, #2211), rpS6 (Cell Signalling, #2217), p-4E-BP1 (ser65) (Cell Signalling, #9451), 4E-BP1 (Sigma, #SAB4500736), actin (sc-56,459). The membranes were subsequently washed three times with TBST before incubation for 2 h with the respective horseradish peroxidise (HRP) conjugated secondary antibodies at room temperature. The membranes were developed with Amersham ECL western blotting detection system (Amersham) and light signals were detected on X-ray film. Lastly, densitometry analysis of western blots was performed using Quantity One software (Bio-rad).

2.7. Assessment of melanocyte density

Zebrafish embryos at 3 dpf were euthanised with 10% benzocaine and de-chorionated mechanically prior to visualisation with an inverted microscope (Carl Zeiss). Images of lateral melanocyte pigmentation of rapamycin or vehicle (DMSO) treated zebrafish embryos were captured using IS Capture software with a digital video camera mounted on the Zeiss inverted microscope. Melanocyte density was quantitated using the colour threshold selection tool in ImageJ software version 1.49, by referring to a protocol described by Tarafder et al. [32].

2.8. Whole mount SA-β-Gal staining and quantification

Zebrafish embryos (5 dpf) were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4 °C and then washed three times for 15 min each with 1 × PBS. Then, fixed embryos were incubated in β-Gal staining solution (#9860; Cell Signalling), pH 6.0 for