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

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A B S T R A C T

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 miRNAs 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 eIF-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.

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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].
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’t 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) animal model in studying complex biological processes, as well as for modulating the magnitude of gene expressions in organisms [21].

The development of angiogenesis in mice and zebra fish miRNAs are highly conserved [20] and been reported to be de-regulated in pathological conditions [23,24]. while, miR-34 was found to be up-regulated during aging [27]. These new molecular regulators have mechanisms regulating 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 NaHCO3) 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 embryos 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, #AB4500736), actin (sc-56,459). The membranes were subsequently washed three times with TBST before incubation for 2 h with the respective horseradish peroxidase (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 Tairafer 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
16 h at 37 °C. Stained embryos were washed three times for 15 min each time with 1 × PBS. All embryos were photographed under the same conditions using Canon Powershot A640 Digital Camera affixed to a stereomicroscope (Olympus, SZX10). SA-β-gal activity in each embryo was quantitated using the color threshold selection tool in ImageJ software version 1.49 at the regions that showed visually positive SA-β-gal staining [33]. SA-β-gal activity in the yolk region was excluded from the colour threshold selection [34]. The selected area of positively staining was normalised to total area and quantified in percentages.

2.9. Whole mount ALP staining and microscopic evaluation

Zebrafish embryos (3 dpf) were fixed in 4% PFA in PBS overnight at 4 °C, then permeabilised and dehydrated in 50% methanol prior to overnight incubation in 100% methanol at −20 °C. Next, dehydrated embryos were incubated in pre-chilled acetone at −20 °C prior to double washes with 1 × PBS Tween (PBST) for 5 min each. Embryos were equiv- librated or bathed 3 times with NTMT buffer (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM MgCl2, 0.1% Tween 20) at room temperature for 10 min each before staining with 9 μL/mL NBT–BCIP solution (#1168145001; Roche) at room temperature for 30 min. Lastly, stained embryos were washed 3 times with 1 × PBST for 5 min each before mounting with 87% glycerol and imaging using a Canon Powershot A640 Digital Camera affixed to a stereomicroscope (Olympus, SZX10). Anti-angiogenic response was evaluated by assessing the intersegmental vessels (ISV) and sub-intestinal vessels (SIV) following these criteria: (a) defective formation of complete ISV [35], (b) absence of vessels spiking out of the SIV basket, (c) shrinking of the SIV basket with less than 50% deformation of complete ISV [35]. SA-β-gal activity in the yolk region was excluded from the colour threshold selection [34]. The selected area of positively staining was normalised to total area and quantified in percentages.

2.10. Quantification of microRNA expression by quantitative RT-PCR

Two-step quantitative RT-PCR was used to quantify miRNA expression levels. cDNA was synthesised from 10 ng of total RNA using miCURY LNA™ Universal RT microRNA PCR Universal CDNA Synthesis Kit II (Exiqon), followed by second step of LNA™ real time PCR amplification using miCURY LNA™ Universal RT microRNA PCR ExiLENT SYBR® Green Master Mix (Exiqon), miCURY LNA™ Universal RT microRNA PCR LNA™ PCR Targeted miRNA primers were dre-miR-124-3p ( #2,107,549), dre-miR-140-5p (annotated in miBase 20) ( #204,540), dre-miR-204 (annotated in miBase 20) (#206,072), dre-miR-143 (annotated in miBase 20) (#205,992), dre-miR-9-5p (annotated in miBase 20) (#204,540), dre-miR-7a (annotated in miBase 20) (#205,877), dre-miR-25-3p (#2,111,968), dre-miR-9-3p (#2,114,469). Dre-miR-30b-5p (annotated in miBase 20) (#204,765) was used as endogenous reference miRNA for normalisation. Respective fold changes were calculated by the ∆∆CT method.

2.11. Statistical analysis

All data were presented as mean ± standard deviation (SD), and considered statistically significant when P < 0.05. Analyses of statistical significance were performed using unpaired Student’s t-test or one-way analysis of variance (ANOVA) tests, where relevant, using Graphpad Prism v6.0 software (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Rapamycin mitigates zTOR signalling pathway in zebrafish embryo

To investigate the function of the zebrafish target of rapamycin (zTOR), zebrafish embryos were treated with the inhibitor of TOR protein, rapamycin (400 nM) as previously reported [19]. Its effect on the expressions of downstream proteins of the zTOR pathway were validated by immunoblotting (Fig. 1). Rapamycin treatment at 4 hpf down-regulated the expression of phosphorylated protein P70S6 kinase (p-P70S6K) (Fig. 1A) and completely suppressed an effector protein further downstream, phosphorylated ribosomal protein S6 (p-rpS6) (Fig. 1C). Meanwhile, rapamycin treatment at 8 hpf down-regulated the expressions of both p-P70S6K and p-rpS6 as well as another downstream effector of zTOR, p-4E-BP1 (Fig. 1E).

3.2. Rapamycin treatment leads to melanocyte suppression in zebrafish embryos

Melanocytes are developed from neural crest cells [37]. They begin to appear at 24 hpf and their numbers increase rapidly up to approximately 60 hpf [38]. The neural crest-derived melanocytes reach the dorsal, ventral, yolk and lateral stripes at 3 dpf. These cells provide an excellent model for developmental study and were used in the present study to determine the extent of rapamycin inhibition on embryonic development. At 400 nM, rapamycin treatments commencing from 4 hpf and 8 hpf significantly suppressed the development of lateral stripe melanocytes (Fig. 2A). The reductions were more than 2-fold as indicated by the melanocyte pixel densities when compared to that of the vehicle control (Fig. 2B).

3.3. Mitigation of zTOR diminishes in vivo SA-β-Gal activity

Cytochemically and histochemically detectable senescence-associated beta galactosidase (SA-β-gal) activity at pH 6.0 allows the differentiation of senescent cells from young cells in cell culture and mammalian tissues [39] and this has been widely used as a biomarker of cellular senescence in vertebrate animal systems [40,41]. We adopted an experimental end-point of 5 dpf to avoid potential spurious effects due to caloric restriction as well as other nutritional deficiencies, as this would be the point the larvae begin to rely on oral nutritional intake in addition of yolk utilisation [34]. Areas of stained yolk were also excluded from quantification due to differences in initial yolk volume and yolk consumption over time [34]. The assay demonstrated that SA-β-gal-positive staining was decreased following rapamycin treatment (Fig. 3A–D). Embryos treated from 4 hpf and 8 hpf displayed an approximately half fold decrease SA-β-gal activity staining (Fig. 3E) in the head and dorsal trunk compared to their respective controls.

3.4. Mitigation of zTOR attenuates in vivo angiogenesis

Alkaline phosphatase (ALP) staining is an accepted method for staining endothelial cells within blood vessels due to the relatively high levels of endogenous alkaline phosphatase activity [42] and is best applied in embryos up to 3 dpf [43]. At 3 dpf, the SIV which originate from the ducts of cuvier at 48 hpf are completely formed [44]. SIV as-
rapamycin treatment, a panel of miRNAs were selected for qRT-PCR based on their known correlations with senescence [9,48], angiogenesis [49–51] and the mTOR pathway [52,53]. Out of the seven miRNAs studied, we observed three, which showed significant up-regulation in rapamycin-treated zebrafish embryos at 4 hpf, namely dre-miR-9 (both 5p and 3p arms), dre-miR-25-3p and dre-miR-124-3p. In particular, dre-miR-9-5p had an approximately 2-fold up-regulation. However, none of the miRNAs showed de-regulation at treatment beginning from 8 hpf (Fig. 5).

4. Discussion

Although the involvement of mTOR signalling pathway in regulating senescence and angiogenesis has been extensively investigated in recent years, studies on the roles of miRNAs as molecular drivers of signalling molecules upstream and downstream of mTOR are still rudimentary. It is also unclear if zTOR is involved in similar biological functions in the zebrafish even though it is an orthologue of mTOR. In the present study, we demonstrated that suppression of zTOR is associated with physiological changes, including reduced embryonic senescence and melanogenesis and decreased angiogenic response. These physiological changes were accompanied by the significant up-regulation of several zTOR-associated miRNAs.

Treatment with rapamycin led to the inhibition zTOR pathway as evidenced by the suppression of the phosphorylation of P70S6K, rpS6 and 4E-BP1. zTOR is expressed ubiquitously during early embryogenesis in zebrafish [54]. However, its expression is spatially and temporally dynamic. Fleming et al. demonstrated that zTOR is expressed in the head...
and developing gut [19, 54] from 35 to 57 hpf. Another study reported zTOR gene expression at 6 hpf, 18 hpf and 24 hpf [30]. Rapamycin-treated embryos at very early embryogenesis (1 cell stage) underwent the normal developmental sequence, albeit with a mild delay [55]. Therefore, rapamycin treatment commencing from 4 hpf and 8 hpf respectively in the present study was expected to cause effective suppression in zTOR activity without inducing overt defects. Our results were consistent with a previous study that demonstrated a 30% reduction of phospho-P70S6K/total P70S6K expression level in embryos treated between 0 and 3 hpf [19] and complete reduction of phospho-

Fig. 2. Inhibition of zTOR leads to melanocyte suppression. (A) Representative images show suppressed melanocyte formation at 3 dpf in 400 nM rapamycin-treated embryos. Melanophore cells at the lateral stripe (arrows). (B) Quantification analysis demonstrates melanocyte pixel density was diminished for both 4 hpf and 8 hpf rapamycin treatments. Scale bar: 400 μm. (Data represent the mean ± SD, experiments were done in triplicate, n = 30 each replicate). Statistical significance is expressed as ***/P < 0.0001 (Student’s t-test) versus DMSO vehicle control.

Fig. 3. Mitigation of zTOR diminishes embryonic SA-β-gal activity. Embryos treated with 400 nM rapamycin from 4 hpf (B) or 8 hpf (D) to 5 dpf demonstrated lower SA-β-gal activity than their respective 0.04% DMSO vehicle control (A, C). Top panel: Lateral imaging of embryos with encircled yolk areas (white dotted line) excluded from SA-β-gal quantification, as recommended by Kishi et al. [34]. Bottom panel: Larger view of four SA-β-gal stained embryos. SA-β-gal quantification analysis (E) expressed as stained pixel density shows a significant decrease of SA-β-gal activity with rapamycin treatment at both 4 hpf and 8 hpf. Scale bars: 300 μm (top panel), 500 μm (bottom panel). (Data represent the mean ± SD, experiments were done in triplicate, n = 5 each replicate). Statistical significance is expressed as ***/P < 0.001 (Student’s t-test) versus DMSO vehicle control.
Fig. 4. Mitigation of mTOR suppresses angiogenesis response in zebrafish embryo. Embryos treated with 400 nM rapamycin from 4 hpf (B) or 8 hpf (D) to 72 hpf showed inhibited angiogenesis compared to their respective 0.04% DMSO vehicle controls (A,C). Top panel: Lateral view of ALP stained embryos showing both the SIV (arrows) and ISV (inset, arrows). ISV appeared normal in vehicle control (A,C) as compared to defected ISV in rapamycin-treated embryo (B, D). Bottom panel: Dorsal view of SIV for the vehicle control, with the SIV appearing as a smooth basket-like structure (A, C) and rapamycin-treated embryo, with the SIV appearing tortuous (B, D). ALP quantification analysis (E) expressed as stained ISV pixel density showed a significant suppression of angiogenic response following rapamycin treatment at 4 hpf and 8 hpf. Scale bars: 250 μm (top panel), 150 μm (bottom panel). (Data represent the mean SD, experiments were done in triplicate, n = 5 each replicate). Statistical significance is expressed as **P < 0.01, ****P < 0.0001 (Student’s t-test) versus DMSO vehicle control.

Fig. 5. qRT-PCR validation of the expression of miRNAs in rapamycin-treated embryos. Quantification analysis shows the relative expression fold change of dre-miR-7-5p, dre-miR-9-3p, dre-miR-9-5p, dre-miR-25-3p, dre-miR-124-3p, dre-miR-140-3p, dre-miR-140-5p, dre-miR-143-3p and dre-miR-204-5p. There was significant up-regulation of dre-miR-9-3p, dre-miR-9-5p, dre-miR-25-3p and dre-miR-124-3p following rapamycin treatment at 4 hpf. Bars represent fold change normalised to dre-miR-30b-5p which is the reference miRNA of their respective controls. (Data represent the mean ± SD, n = 3). Statistical significance is expressed as *P < 0.05, **P < 0.01 (one-way ANOVA) versus DMSO vehicle control.
rpS6/tot rpS6 in rapamycin-treated zebrafish at 72 hpf after 24 h of treatment from 48 hpf [56]. However, there was less down-regulation of P70S6K seen in embryos treated from 8 hpf as compared to embryos treated from 4 hpf, possibly due to the shorter total exposure time to rapamycin for the latter group. A recent study showed that rapamycin treatment for 2 days decreased phosphorylation of 4E-BP1 in zebrafish adults [7] but not in embryos. Interestingly, in the present study, down-regulation of 4E-BP1 was not observed in the embryos treated with rapamycin from 4 hpf. This is likely because rapamycin could differentially inhibit P70S6K and 4E-BP1 as reported by Choo et al. [57]. Hence, in the present study, down-regulation of both downstream effectors of zTOR observed after rapamycin treatment during early embryogenesis suggests that suppression of the zTOR pathway was evident and could be further applied for downstream functional studies.

TOR signalling is known to play an important role in embryonic development. Here, we examined the effect of the inhibition of TOR in zebrafish embryos on melanocyte development, senescence and vessel sprouting. Melanocytes are developed from the neural crest lineage and the genetic network regulating melanocyte differentiation is modulated by transcription factors and extracellular signals [58,59]. Inhibition of TOR in the inhibition of melanogenesis similar to that observed by Moriyama et al. where rapamycin treatment resulted in significant suppression of melanocyte formation on the lateral sides of rapamycin-treated Xenopus embryos [60]. However, several studies have reported that inhibition of mTOR stimulates melanogenesis in vitro [61,62]. While several pathways including extracellular signal regulated kinases (ERK), microphthalmia-associated transcription factor (MITF) and AKT are known to be essential in modulating melanocyte formation [62], the extent of the involvement of zTOR in melanogenesis remains to be investigated.

Caloric restriction could prevent senescence and to prevent spurious senescence inhibition effects resulting from caloric restriction, embryos at 4 and 8 hpf were used. Developing embryos are wholly dependent on their yolk sac, with steady depletion in yolk sac volume seen from 4 dpf to 7 dpf as embryos transition into larvae and continue to grow [63]. In the whole mounted stained embryos, we showed that zTOR inhibition was associated with decreased SA-β-gal activity, a cellular senescence marker that is also used for organism aging studies [33]. This observation suggests that inhibition of zTOR delays embryonic senescence and is in agreement with earlier reports of attenuation of senescence by the inhibition of mTOR in various cell lines [64,65] and in prolonging the life span of mice, C. elegans and D. melanogaster [66–68].

Xue et al. reported that mTOR inhibition during the first 12 days of VEGF-A induced 4- to 6-weeks-old mice results in blocked angiogenesis at the ears of the animals [8]. In addition, abrogation of mTOR signalling pathway also led to anti-angiogenic effects in C57BL/6 mice [69]. Moreover, Sasore et al. showed anti-angiogenic effects on ISV development in zebrafish larvae using PI3K/Akt/mTOR inhibitors [30]. Here, we observed that suppression of zTOR pathway was accompanied with defective formation of both the SIVs and ISVs, suggesting that zTOR is important for vascular development in zebrafish.

miRNAs function as transcriptional regulators of mRNA by binding to one or more mRNA transcripts and de-stabilize target transcripts and inhibit protein translation. In developing organisms, complex temporal cues control the generation of body structures on schedule [70]. miRNAs such as lin-4 and let-7 have been shown to exhibit temporal expression changes to control their target genes that dictates cell fate and the progression to the next developmental stage in C. elegans [71,72] and D. melanogaster [73]. In the present study, the expressions of dre-mir-9 (both 5p and 3p arms), dre-mir-25-3p and dre-mir-124-3p were significantly up-regulated at 4 hpf but not 8 hpf following rapamycin treatment. miRNAs have the capacity to regulate gene expression in response to changing environmental stimuli by controlling protein synthesis in a highly specific spatiotemporal pattern [74]. A previous study has demonstrated that miR-22 and miR-185 were significantly up-regulated after 8 h of co-culture with platelets. However, treatment after 1 h, 3 h and even 24 h did not show any significant up-regulation [75]. In the present study, temporal miRNA expression changes observed at certain time-points after the rapamycin treatment reflect the biological responses of the developing embryos to rapamycin treatment, where differential miRNA expressions play a role to down- or up-regulate target gene expressions which could facilitate cell repair or adaptation to the treatment for survival. In developing Xenopus embryos, TOR and raptor have been shown to be consecutively expressed throughout the developmental stages of unfertilized egg to larva stage, and TORC1 activity was effectively inhibited by rapamycin in a dose-dependent manner that clearly delayed the speed of development [60,76]. This suggests that TORC1 signalling is vital during early developmental stages. The zebrafish results corroborate the critical requirement for TOR during different stages of embryonic development, by having rapamycin treatment beginning from 4 hpf, which is at the blastula stage, differentially altering the expression of miRNAs as compared to rapamycin treatment beginning at 8 hpf, wherein the epiboly stage is nearing completion and there is initiation of morphogenesis.

Our findings are in agreement with previous reports of inhibition of mTOR and Akt signalling that led to suppressed cell proliferation by over-expression of miR-124 [52]. In addition, miR-124 is also known to modulate cellular senescence through tumour suppressor activity [77] and inhibit angiogenesis in glioma [49] whereas the role of miR-9 in angiogenesis and senescence is less straightforward as there are contradicting reports. Over-expression of miR-9 was found to inhibit angiogenesis in neuroblastoma cells [51,78] and in HepG2 cells [79]. In contrast, it was reported that secretion of miR-9 by tumour cells stimulates angiogenesis in endothelial cells via the JAK-STAT pathway [80]. We showed that miR-9 was up-regulated in association with decreased embryonic SA-β-gal activity in vivo, but it was found up-regulated in senescent endothelial cell [81]. These suggest that miR-9 may exhibit intrinsic cellular activities or could be a result of its involvement in modulating diverse signalling pathways. Similarly, the miR-106b – 25 cluster which consists of miR-25 has been described as playing a central role in the regulation of angiogenic properties [50] and inhibition of cell senescence [48]. A previous study showed that down-regulation of miR-25 led to senescence in fibroblasts via MAP2K4 pathway [9]. On the other hand, Suh et al. reported that miR-25 enhanced mTOR activity in glioblastoma cells [53].

Many genes, such as DNA repair genes, directly respond to DNA damage caused by drug exposure whereas miRNAs, which act as post-transcriptional regulators for gene expression, respond indirectly via the alteration of gene expression [82]. It was also reported that miRNA expression levels do not always reflect its actual activity [83,84]. In the present study, reduced angiogenesis and senescence at 8 hpf after treatment with rapamycin could be due to a direct response to zTOR inhibition rather than a direct effect of the expression of the particular miRNA. The identified miRNAs may possibly be regulators of zTOR mRNA expression and would have exhibited differential expression in response to zTOR inhibition. Alternatively, these miRNAs may have other unknown target genes associated with the zTOR pathway as well as pathways regulating senescence and angiogenesis. Moreover, one target gene may be regulated by several miRNAs, which would not have been reflected in our limited miRNAs panel. Our findings suggest the involvement of zTOR in embryonic senescence and angiogenesis which could be potentially mediated by the identified miRNAs. Further investigation by specifically targeting these miRNAs for knockouts will be able to yield details on their impact on zTOR.

In summary, findings from the present study revealed that inhibition of zTOR pathway affects melanogenesis, embryonic senescence, and angiogenesis. This inhibition was accompanied by the de-regulation of dre-mir-9-5p and 3p, dre-mir-25-3p and dre-mir-124-3p. Identification of molecular drivers such as miRNAs in the regulation of senescence, angiogenesis or other developmental processes in vivo would provide a more holistic understanding of the mechanism of these processes as well as new targets for innovative monitoring and therapeutic strategies for age-related vascular pathology.
Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References


