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Title

The role of PI3K/Akt/mTOR signaling in dose-dependent biphasic effects of glycine on vascular development

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Highlights

Inhibition of PI3K/Akt/mTOR signaling impairs vascular development.

Low-glycine induced angiogenesis is blocked by inhibition of mTOR signaling.

High-glycine induced anti-angiogenesis is enhanced by inhibition of mTOR signaling.

Combination of glycine and an mTOR inhibitor affects expression of *VEGF* and *NOS* genes.

Abstract

Glycine, a non-essential amino acid, exerts concentration-dependent biphasic effects on angiogenesis.

Low-doses of glycine promote angiogenesis, whereas high-doses cause anti-angiogenesis. The

phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling

participates in angiogenesis of both physiological development, and pathological events including

tumour and inflammation. We assessed the role of PI3K/Akt/mTOR signaling in vascular

development, and the interaction with glycine, using transgenic zebrafish *Tg(fli1a:Myr-mCherry)^{ncv1}*

embryos expressing fluorescent proteins in vascular endothelial cells. Treatment with inhibitors of

mTORC1 (rapamycin and everolimus), mTORC1/mTORC2 (KU0063794), PI3K (LY29400), and Akt

(Akt inhibitor) decreased the development of intersegmental vessels (ISVs). These inhibitors cancelled

the angiogenic effects of a low-dose of glycine, while acted synergistically with a high-dose of glycine

in anti-angiogenesis. mTOR signaling regulates the gene expression of *vascular endothelial growth*

factor (VEGF), a major angiogenic factor, and *nitric oxide (NO) synthase (NOS)*, an enzyme for the

synthesis of an angiogenic mediator NO. Expressions of *VEGF* and *NOS* were consistent with the vascular features induced by glycine and an mTOR inhibitor. Our results suggest that PI3K/Akt/mTOR signaling may interact with dose-dependent biphasic effects of exogenous glycine on *in vivo* angiogenesis. mTOR signaling is a key target for cancer therapy, thus, the combining mTOR inhibitors with glycine may be a potential approach for controlling angiogenesis.

Keywords

Glycine; angiogenesis; PI3K; Akt; mTOR; zebrafish

Introduction

Angiogenesis is the formation process of new vascular network from pre-existing vessels into an avascular area [1], and is critical for both physiological (e.g., embryogenesis and wound healing) and pathological events (e.g., chronic inflammation and tumour growth) [2] [3]. Therefore, it is important to understand the mechanisms regulating angiogenesis.

Glycine is a non-essential amino acid, and displays effects in inhibitory neurotransmission, cell proliferation, and cell protection [4] [5] [6]. Recently we demonstrated that glycine exerts dose-dependent biphasic effects on vascular development [7]. Low concentrations of glycine promoted the vascular formation of zebrafish embryos, whereas high concentrations reduced it. These angiogenic and anti-angiogenic effects of glycine appear to generally rely on glycine transporters and receptors.

VEGF plays a central role in angiogenesis, which drives endothelial cell functions including survival, proliferation, migration, and the formation of vascular structure [8] [9] [10]. Loss of VEGF signaling caused vascular defects in zebrafish embryos [11] [12]. NO generated by NOS stimulates angiogenesis and vascular permeability directly or by interacting with VEGF [13,14] [15]. The biphasic effects of glycine in zebrafish vasculature were accompanied by the alterations of gene expression of *VEGF* and *NOS* genes [7].

PI3K/Akt/mTOR signaling is required for numerous cellular and organ functions, including survival, proliferation, metabolism, and homeostasis [16], and is also responsible for regulating angiogenesis [17]. mTOR, a serine-threonine kinase, binds with some subunit proteins and forms two functional complexes,

mTOR complex 1 (mTORC1) containing mTOR and RAPTOR, and mTOR complex 2 (mTORC2) containing mTOR and RICTOR. There is a complex interplay among PI3K, AKT, mTORC1 and mTORC2. PI3K/Akt inhibits tuberous sclerosis complex (TSC), an mTORC1 suppressor, and thereby activates mTORC1. While, Akt is phospho-activated by mTORC2, leading to mTORC1 activation. Our previous studies and others have revealed that endothelial cell growth and the formation of vessel structures are interrupted by pharmacologic or genetic disruption of mTOR signaling [18] [19,20]. Besides these functions in endothelial cells, the role of mTOR signaling in gene expression is well established. Upon angiogenic stimuli, such as hypoxia, the expression of the *VEGF* gene is induced by the hypoxia-inducible factor1 α (HIF-1 α) transcription factor, leading to angiogenesis [17] [21]. mTORC1 drives the synthesis of HIF-1 α . Akt also contributes to the activation of endothelial NOS, which produces NO. Thus, mTORC2 is related to induce of VEGF and NO through activation of Akt. Thus, in the present study, we evaluated the interaction between PI3K/Akt/mTOR signaling with glycine on angiogenesis using zebrafish embryo models.

Material and methods

Materials

Glycine (Cat. No. G7126; Sigma–Aldrich, St. Louis, MO, USA), rapamycin (Cat. No. 184-01311; Wako Pure Chemical Industries, Osaka, Japan), a rapamycin derivative everolimus (Cat. No. S1120; Selleck Chemicals, Houston, TX, USA), KU0063794 (Cat. No. 510-90421; Wako), LY294002 (Cat. No. 129-04861; Wako), and Akt inhibitor VIII, isozyme selective (Cat. No. 124018; Calbiochem La Jolla, CA, USA) were used.

Zebrafish

Zebrafish experiments were approved by the institutional guidelines established by the Committee on Animal Experimentation of Hokkaido University (Sapporo, Japan) (approval no. 16-0084). A transgenic zebrafish line *Tg(fli1a:Myr-mCherry)^{ncv1}* [22], which has been used for studying vascular development [23], was obtained from the National BioResource Project (Japan). Adult zebrafish were maintained under a 14 h light/10 h dark cycle at 28 °C, and fed TetraMin (Tetra, Melle, Germany) and hatched brine shrimp (Salt lake), as previously described [24,25]. Embryos obtained by mating male and female fish were maintained in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄; pH 7.4). For experiments, embryos were randomly placed and maintained in a 24-well plate (10 embryos per well) with 500 µL of E3 solution, and exposed to glycine at 3 h post

fertilization (hpf), and treated with inhibitors at 9 hpf. Survival rates were calculated at 24 hpf. After the dechoriation with 0.025% actinase E (Cat. No. 29003-64; Nacalai Tesque, Kyoto, Japan) and euthanization using tricaine (Cat. No. E10521; Sigma–Aldrich) and cooling, embryos at 28 or 30 hpf were fixed with 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C.

Analysis of vascular development

Images of ISVs in the midtrunk region of all embryos, except for them destroyed or showing no fluorescence, were acquired using Axio Imager 2 (Zeiss, Germany, Oberkochen) and IX71 (Olympus, Tokyo, Japan) fluorescence microscopes. Brightness and contrast were uniformly adjusted across the entire images using ImageJ software version 1.44o (National Institutes of Health, Bethesda, MD, USA). The number and length of ISVs were blindly counted and measured on the fluorescence images. ISV length measurements were performed on five vessels per one embryo by manually tracing each vessel structure using ImageJ software.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of whole zebrafish embryos was extracted with QIAzol Lysis Reagent (Cat. No. 79306; Qiagen, Venlo, Netherlands), followed by the RT reaction using an PrimeScript RT Master Mix (Cat.

No. RR036A, Takara Bio Inc., Shiga, Japan). PCR was performed using a Taq PCR Core Kit (Ca. No. 201223; Qiagen) in a standard thermal cycler PC707 (ASTEC, Fukuoka, Japan) under the thermal cycling conditions of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s. The following primers were used: *vegfaa*, fwd 5'-CTCCTCCATCTGTCTGCTGTAAAG-3' and rev 5'-CTCTCTGAGCAAGGCTCACAG-3' (product size: 490 bp); *nos2a*, fwd 5'-GTGTTCCCTCAGAGAACAGAT-3' and rev 5'-GATCAGTCCTTTGAAGCTGAC-3' (product size: 822 bp); and *elfa*, fwd 5'-CTTCTCAGGCTGACTGTGC-3' and rev 5'-CCGCTAGCATTACCCTCC-3' (product size: 358 bp). After the electrophoresis of PCR products at a 1.5% agarose gel, PCR bands were visualized with SYBR Gold Nucleic Acid Gel Stain (Cat. No. S11494; Molecular Probes, Eugene, OR, USA) and detected under a UV transilluminator (Red Imaging System; Alpha Innotech, San Diego, CA, USA). Quantification of the PCR band intensity was blindly performed by densitometry using the ImageJ software and normalized to the levels of *elfa*.

Statistical analysis

Experimental results are indicated as the mean $\pm$ SEM (analysis of vascular development) or SD (Semi-quantitative RT-PCR). Statistical significance among multiple groups was determined by Dunnett's test using the MEPHAS webtool

(<http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/dunnett-e.html>). F-tests followed by Student's

t-tests were used for comparisons of two groups. The level of significance was set at $p < 0.05$.

Results

Inhibition of PI3K/Akt/mTOR signaling impairs vascular development of zebrafish embryos

Firstly, we assessed the contribution of mTOR signaling to vascular development *in vivo* using transgenic zebrafish *Tg(fli1a:Myr-mCherry)^{ncv1}* embryos. Embryos were treated with mTORC1 inhibitors (rapamycin and everolimus) and a dual mTORC1/mTORC2 inhibitor (KU0063794) at 5, 10, and 50 μ M concentrations from 9 hpf, and fixed at 30 hpf, followed by observation of vascular structures in the midtrunk region. About 20 to 36 hpf, ISVs start to sprout from dorsal aorta and reach to and form the dorsal longitudinal anastomotic vessels (DLSVs) [26,27]. In agreement with the previous reports, ISVs sprouted, elongated and partially formed DLAVs in embryos treated with 0.1% DMSO control (Fig. 1A). Significant reductions in the number and length of ISVs were shown after treatment with mTOR inhibitors at 5, 10, and 50 μ M concentrations, compared with the control (Fig. 1 A, B and C). Embryos treated with these inhibitors had a high survival rate as well as DMSO control

(Fig. 1D).

Next, we treated zebrafish embryos with a PI3K inhibitor (LY294002) and an Akt inhibitor (Akt inhibitor) at 5, 10, and 50 μ M concentrations from 9 hpf, and analysed the vascular structure at 30 hpf. There was a significant difference in the number of ISVs among embryos treated with 5 and 10 μ M LY294002 and 5, 10, and 50 μ M Akt inhibitor (Fig. 2A and B). At these concentrations, inhibitors caused a significant reduction in ISV length compared with 0.1% DMSO (Fig. 2A and C). Although embryos treated with 5 and 10 μ M LY294002 and 5, 10, and 50 μ M Akt inhibitor had a high survival rate, the treatment with 50 μ M LY294002 led to severe developmental defects and death (Fig. 2D). These results in Fig. 1 and Fig. 2 suggest that the activation of PI3K/Akt/mTOR signaling is required for vascular development in zebrafish embryos.

Inhibition of PI3K/Akt/mTOR signaling interacts with effects of glycine on vascular development

Low concentrations of glycine promotes the development of ISVs in zebrafish embryos [7]. We examined whether PI3K/Akt/mTOR signaling contributes to the vascular development induced by a low concentration of glycine (10 mM). At 3 hpf, we exposed embryos to 10 mM glycine, followed by

treatment with 10 μ M LY294002, Akt inhibitor, rapamycin, everolimus or KU0063794 at 9 hpf, and observed the vascular structure at 28 hpf. Consistent with our previous report [7], although exposure to 10 mM glycine did not affect the number of ISVs, it increased ISV length (Fig. 3A and B). In the presence of 10 mM glycine, PI3K/Akt/mTOR inhibitors did not decrease the number of ISVs (Fig. 3A), but blocked the increase in ISV length induced by 10 mM glycine alone (Fig. 3B). Thus, glycine appears to reverse the reduced number and length of ISVs induced by inhibition of PI3K/Akt/mTOR signaling (Fig. 1, 2, 3A and 3B). High survival rates were recorded in these treatment groups (Fig. 3C).

High concentrations of glycine impairs the development of ISVs [7]. Next, we assessed the interaction of PI3K/Akt/mTOR signaling on the inhibitory effects caused by a high concentration of glycine (400 mM). Embryos were exposed to 400 mM glycine at 3 hpf, treated with 10 μ M LY294002, Akt inhibitor, rapamycin, everolimus or KU0063794 at 9 hpf, and observed at 30 hpf. Embryos exposed to 400 mM glycine showed the decrease of ISV length, but not ISV number (Fig. 3D and E). Some combinations of 400 mM glycine and inhibitors caused the further decrease of the number and length of ISVs, compared with 400 mM glycine alone (Fig. 3D and E). The survival rates remained unaffected in combinations of 400 mM glycine and inhibitors (Fig. 3F).

These results indicate that a low concentration of glycine may be dependent on or interact with PI3K/Akt/mTOR signaling, leading to promote vascular development. On the other hand, the inhibitory effects occurred with a high concentration of glycine appears to be synergistic with PI3K/Akt/mTOR signaling.

Combination of glycine and an mTOR inhibitor affects expression of *VEGF* and *NOS* genes.

PI3K/Akt/mTOR signaling positively modulates angiogenesis through upregulation of angiogenic factors, including VEGF and NO [17]. We examined the expression of *vegfaa* and *nos2a* genes in embryos exposed to glycine for 3–30 hpf and an mTOR inhibitor for 9–30 hpf. Consistent with the angiogenic effects of glycine (Fig. 3B), embryos exposed to 10 mM glycine showed the increased expression of *vegfaa* and *nos2a* genes (Fig. 4A). The increase induced by 10 mM glycine was prevented by the addition of 10 μ M KU0063794 (Fig. 4B). Exposure to 400 mM glycine downregulated the expression of *nos2a*, but not *vegfaa* (Fig. 4C). Combination of 400 mM glycine and 10 μ M KU0063794 decreased the expression of *vegfaa*, and further reduced the decreased expression of *nos2a* caused by 400 mM glycine alone (Fig. 4D). These findings indicate that VEGF and NOS may be involved in the vascular response to treatment with glycine and an mTOR inhibitor.

Discussion

We previously revealed that glycine exerts dose-dependent biphasic effects on angiogenesis *in vivo* [7].

In the present report, we demonstrated the role of PI3K/Akt/mTOR signaling in vascular development, and in biphasic effects of glycine on angiogenesis. Inhibition of PI3K/Akt/mTOR signaling blocked vascular elongation promoted by a low concentration (10 mM) of glycine. A high concentration (400 mM) of glycine reduced vascular development, and further exacerbate it when combined with inhibitors of PI3K/Akt/mTOR signaling. These effects were accompanied by alterations of the expression of *VEGF* and *NOS* genes.

mTOR signaling is one of the crucial pathway regulating endothelial cell functions including survival, proliferation, migration, elongation, and vascular assembly, and these endothelial functions are blocked by disruption of mTOR signaling [20,21] [10,18,19]. Mouse pulmonary endothelial cell growth stimulated by VEGF was decreased upon loss of RAPTOR or RICTOR, a subunit of mTORC1 or mTORC2 respectively [20]. The proliferations of mouse endothelial cell lines were inhibited by the treatment with everolimus or KU0063794 [19]. Although loss of RAPTOR did not affect vascular

assembly, loss of RICTOR impaired it [20]. Furthermore, RICTOR deficiency in endothelial cells inhibited tumour growth and tumor-angiogenesis *in vivo* [20]. Mouse embryonic stem cells-derived endothelial cells treated with KU0063794, but not everolimus, failed to elongate and form vessel structure [18]. In addition to these functions in endothelial cells, mTOR signaling has been known to regulate the production of angiogenic factors [17] [21]. mTORC1 is related to induction of VEGF, mTORC2 is involved in increase of VEGF and NO. These reports appear to suggest the more contribution of mTORC2 in angiogenesis, in addition to mTORC1. Therefore, we had predicted that a dual mTORC1/mTORC2 inhibitor would be more effective for anti-angiogenesis. Nonetheless, our present study showed a similar significant reduction of vascular development in mTORC1-specific inhibitors and the dual mTORC1/mTORC2 inhibitor (Fig. 1). Although mTORC2 is thought to be not sensitive to rapamycin and its derivatives including everolimus, has been reported to be inhibited by the long-term treatment to certain cell types [28]. Thus, further studies should be needed for characterization of the exact efficacy of mTOR inhibitors in *in vivo* angiogenesis.

Treatment with glycine enhanced the phosphorylation of S6 kinase, an mTORC1 effector, and promoted vessel network formation *in vitro* [29]. Oral administration of bitopertin, an inhibitor of glycine transporters, reduced the activation of mTORC1 elevated in β -thalassemia model mice [30]. In

endothelial cells, silencing of *MTHFD2*, an enzyme involved in glycine metabolism, has been reported to reduce intracellular glycine and suppress vascular sprouting [31]. Rapamycin inhibited the expression of the *MTHFD2* gene in endothelial cells [31], indicating that mTOR activation is involved in the synthesis of glycine. Our results demonstrated that inhibition of PI3K/Akt/mTOR signaling blocked the angiogenic effect of a low concentration of glycine, whereas enhanced the anti-angiogenic effect caused by a high concentration of glycine (Fig. 3). Namely, a low concentration of glycine protected vascular development against inhibition of PI3K/Akt/mTOR signaling, while a high concentration acted to impair it. Furthermore, the expression of the *VEGF* and *NOS* genes correlated with the vascular feature caused by glycine with the mTOR inhibitor (Fig. 4).

In this report, we demonstrate that PI3K/Akt/mTOR signaling may contribute to glycine-induced angiogenesis and anti-angiogenesis. Inhibition of PI3K/Akt/mTOR signaling showed the blocking or synergistic effects on concentration-dependent biphasic action of exogenous glycine, although the detailed mechanism remain to be defined. mTOR inhibitors have been validated to reduce tumor-growth and tumor-angiogenesis in experimental research; however, the effects are insufficient in clinical trials [32]. Hence, the combination effects with other agents are expected [21] [33]. Targeting glycine and PI3K/Akt/mTOR signaling may be a beneficial for controlling angiogenesis.

Author contributions

K.T-T conceived the research plans and performed experiments. M.S, M.F and M.T contributed to experiments and data analysis. All authors wrote and reviewed the article.

Declaration of competing interest

The authors declare no competing financial interests.

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References

- [1] R.H. Adams, K. Alitalo, Molecular regulation of angiogenesis and lymphangiogenesis, *Nat Rev Mol Cell Biol.* 8 (2007) 464-478. <https://www.ncbi.nlm.nih.gov/pubmed/17522591>
- [2] R. Cheng, J.X. Ma, Angiogenesis in diabetes and obesity, *Rev Endocr Metab Disord.* 16 (2015) 67-75. <https://www.ncbi.nlm.nih.gov/pubmed/25663658>
- [3] H. Zhong, K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M.M. Georgescu, J.W. Simons, G.L. Semenza, Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics, *Cancer Res.* 60 (2000) 1541-1545. <https://www.ncbi.nlm.nih.gov/pubmed/10749120>
- [4] N.G. Bowery, T.G. Smart, GABA and glycine as neurotransmitters: a brief history, *Br J Pharmacol.* 147 Suppl 1 (2006) S109-119. <https://www.ncbi.nlm.nih.gov/pubmed/16402094>
- [5] J. Van den Eynden, S.S. Ali, N. Horwood, S. Carmans, B. Brone, N. Hellings, P. Steels, R.J. Harvey, J.M. Rigo, Glycine and glycine receptor signalling in non-neuronal cells, *Front Mol Neurosci.* 2 (2009) 9. <https://www.ncbi.nlm.nih.gov/pubmed/19738917>
- [6] Y. Nishimura, J.J. Lemasters, Glycine blocks opening of a death channel in cultured hepatic sinusoidal endothelial cells during chemical hypoxia, *Cell Death Differ.* 8 (2001) 850-858. <https://www.ncbi.nlm.nih.gov/pubmed/11526438>
- [7] K. Tsuji-Tamura, M. Sato, M. Fujita, M. Tamura, Glycine exerts dose-dependent biphasic effects on vascular development of zebrafish embryos, *Biochem Biophys Res Commun.* (2020). <https://www.ncbi.nlm.nih.gov/pubmed/32423801>
- [8] P. Carmeliet, V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoek, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, A. Nagy, Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele, *Nature.* 380 (1996) 435-439. <http://www.ncbi.nlm.nih.gov/pubmed/8602241>
- [9] N. Ferrara, K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K.S. O'Shea, L. Powell-Braxton, K.J. Hillan, M.W. Moore, Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene, *Nature.* 380 (1996) 439-442. <http://www.ncbi.nlm.nih.gov/pubmed/8602242>
- [10] K. Tsuji-Tamura, M. Ogawa, Morphology regulation in vascular endothelial cells, *Inflamm Regen.* 38 (2018) 25. <https://www.ncbi.nlm.nih.gov/pubmed/30214642>
- [11] A. Nasevicius, J. Larson, S.C. Ekker, Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant, *Yeast.* 17 (2000) 294-301. <https://www.ncbi.nlm.nih.gov/pubmed/11119306>

- [12] L.D. Covassin, J.A. Villefranc, M.C. Kacergis, B.M. Weinstein, N.D. Lawson, Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish, *Proc Natl Acad Sci U S A.* 103 (2006) 6554-6559. <https://www.ncbi.nlm.nih.gov/pubmed/16617120>
- [13] R. van der Zee, T. Murohara, Z. Luo, F. Zollmann, J. Passeri, C. Lekutat, J.M. Isner, Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium, *Circulation.* 95 (1997) 1030-1037. <https://www.ncbi.nlm.nih.gov/pubmed/9054767>
- [14] S. Lakshminarayanan, D.A. Antonetti, T.W. Gardner, J.M. Tarbell, Effect of VEGF on retinal microvascular endothelial hydraulic conductivity: the role of NO, *Invest Ophthalmol Vis Sci.* 41 (2000) 4256-4261. <https://www.ncbi.nlm.nih.gov/pubmed/11095623>
- [15] J. Dulak, A. Jozkowicz, Nitric oxide and angiogenic activity of endothelial cells: direct or VEGF-dependent effect?, *Cardiovasc Res.* 56 (2002) 487-488; author reply 489-491. <https://www.ncbi.nlm.nih.gov/pubmed/12445890>
- [16] J.S. Yu, W. Cui, Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination, *Development.* 143 (2016) 3050-3060. <https://www.ncbi.nlm.nih.gov/pubmed/27578176>
- [17] J. Karar, A. Maity, PI3K/AKT/mTOR Pathway in Angiogenesis, *Front Mol Neurosci.* 4 (2011) 51. <http://www.ncbi.nlm.nih.gov/pubmed/22144946>
- [18] K. Tsuji-Tamura, M. Ogawa, Inhibition of the PI3K/Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells, *J Cell Sci.* 129 (2016) 1165-1178. <http://www.ncbi.nlm.nih.gov/pubmed/26826185>
- [19] K. Tsuji-Tamura, M. Ogawa, Dual inhibition of mTORC1 and mTORC2 perturbs cytoskeletal organization and impairs endothelial cell elongation, *Biochem Biophys Res Commun.* 497 (2018) 326-331. <https://www.ncbi.nlm.nih.gov/pubmed/29428724>
- [20] S. Wang, K.R. Amato, W. Song, V. Youngblood, K. Lee, M. Boothby, D.M. Brantley-Sieders, J. Chen, Regulation of endothelial cell proliferation and vascular assembly through distinct mTORC2 signaling pathways, *Mol Cell Biol.* 35 (2015) 1299-1313. <http://www.ncbi.nlm.nih.gov/pubmed/25582201>
- [21] S. Faes, T. Santoro, N. Demartines, O. Dormond, Evolving Significance and Future Relevance of Anti-Angiogenic Activity of mTOR Inhibitors in Cancer Therapy, *Cancers (Basel).* 9 (2017). <https://www.ncbi.nlm.nih.gov/pubmed/29104248>
- [22] S. Fukuhara, J. Zhang, S. Yuge, K. Ando, Y. Wakayama, A. Sakaue-Sawano, A. Miyawaki, N. Mochizuki, Visualizing the cell-cycle progression of endothelial cells in zebrafish, *Dev Biol.* 393 (2014) 10-23. <https://www.ncbi.nlm.nih.gov/pubmed/24975012>

- [23] K. Ando, S. Fukuhara, N. Izumi, H. Nakajima, H. Fukui, R.N. Kelsh, N. Mochizuki, Clarification of mural cell coverage of vascular endothelial cells by live imaging of zebrafish, *Development*. 143 (2016) 1328-1339. <https://www.ncbi.nlm.nih.gov/pubmed/26952986>
- [24] A. Avdesh, M. Chen, M.T. Martin-Iverson, A. Mondal, D. Ong, S. Rainey-Smith, K. Taddei, M. Lardelli, D.M. Groth, G. Verdile, R.N. Martins, Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction, *J Vis Exp*. (2012) e4196. <https://www.ncbi.nlm.nih.gov/pubmed/23183629>
- [25] C. Nüsslein-Volhard, R. Dahm, *Zebrafish : a practical approach*, Oxford University Press, Oxford, 2002.
- [26] S. Isogai, M. Horiguchi, B.M. Weinstein, The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development, *Dev Biol*. 230 (2001) 278-301. <https://www.ncbi.nlm.nih.gov/pubmed/11161578>
- [27] S. Isogai, N.D. Lawson, S. Torrealday, M. Horiguchi, B.M. Weinstein, Angiogenic network formation in the developing vertebrate trunk, *Development*. 130 (2003) 5281-5290. <https://www.ncbi.nlm.nih.gov/pubmed/12954720>
- [28] D.D. Sarbassov, S.M. Ali, S. Sengupta, J.H. Sheen, P.P. Hsu, A.F. Bagley, A.L. Markhard, D.M. Sabatini, Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB, *Mol Cell*. 22 (2006) 159-168. <http://www.ncbi.nlm.nih.gov/pubmed/16603397>
- [29] D. Guo, C.E. Murdoch, H. Xu, H. Shi, D.D. Duan, A. Ahmed, Y. Gu, Vascular endothelial growth factor signaling requires glycine to promote angiogenesis, *Sci Rep*. 7 (2017) 14749. <https://www.ncbi.nlm.nih.gov/pubmed/29116138>
- [30] A. Matte, E. Federti, M. Winter, A. Koerner, A. Harmeier, N. Mazer, T. Tomka, M.L. Di Paolo, L. Defalco, I. Andolfo, E. Beneduce, A. Iolascon, A. Macias-Garcia, J.J. Chen, A. Janin, C. Lebouef, F. Turrini, C. Brugnara, L. De Franceschi, Bitopertin, a selective oral GLYT1 inhibitor, improves anemia in a mouse model of beta-thalassemia, *JCI Insight*. 4 (2019). <https://www.ncbi.nlm.nih.gov/pubmed/31593554>
- [31] J. Hitzel, E. Lee, Y. Zhang, S.I. Bibli, X. Li, S. Zukunft, B. Pfluger, J. Hu, C. Schurmann, A.E. Vasconez, J.A. Oo, A. Kratzer, S. Kumar, F. Rezende, I. Josipovic, D. Thomas, H. Giral, Y. Schreiber, G. Geisslinger, C. Fork, X. Yang, F. Sigala, C.E. Romanoski, J. Kroll, H. Jo, U. Landmesser, A.J. Lusis, D. Namgaladze, I. Fleming, M.S. Leisegang, J. Zhu, R.P. Brandes, Oxidized phospholipids regulate amino acid metabolism through MTHFD2 to facilitate nucleotide release in endothelial cells, *Nat Commun*. 9 (2018) 2292. <https://www.ncbi.nlm.nih.gov/pubmed/29895827>
- [32] J. Baselga, M. Campone, M. Piccart, H.A. Burris, 3rd, H.S. Rugo, T. Sahmoud, S. Noguchi, M. Gnant, K.I. Pritchard, F. Lebrun, J.T. Beck, Y. Ito, D. Yardley, I. Deleu, A. Perez, T. Bachelot, L. Vittori, Z. Xu, P. Mukhopadhyay, D. Lebwohl, G.N. Hortobagyi, Everolimus in postmenopausal

hormone-receptor-positive advanced breast cancer, N Engl J Med. 366 (2012) 520-529. <https://www.ncbi.nlm.nih.gov/pubmed/22149876>

[33] C. Vernieri, F. Corti, F. Nichetti, F. Ligorio, S. Manglaviti, E. Zattarin, C.G. Rea, G. Capri, G.V. Bianchi, F. de Braud, Everolimus versus alpelisib in advanced hormone receptor-positive HER2-negative breast cancer: targeting different nodes of the PI3K/AKT/mTORC1 pathway with different clinical implications, Breast Cancer Res. 22 (2020) 33. <https://www.ncbi.nlm.nih.gov/pubmed/32252811>

Figure legends

Figure 1. Inhibition of mTOR signaling impairs vascular development

Embryos obtained from fluorescent transgenic zebrafish line *Tg(fli1a:Myr-mCherry)^{ncv1}* were treated with mTORC1 inhibitors (rapamycin and everolimus) and an mTORC1/mTORC2 dual inhibitor (KU0063794) at 5, 10, and 50 μ M concentrations from 9 h post fertilization (hpf). Control embryos were exposed to 0.1% DMSO. The vascular structure in the midtrunk region was observed in embryos at 30 hpf. (A) Representative fluorescent images of vascular structure of embryos treated with each inhibitor (10 μ M). Scale bars indicate 100 μ m. Similar results were obtained from three independent experiments. DA, dorsal aorta; ISVs, intersegmental vessels; DLAVs, dorsal longitudinal anastomotic vessels. Arrowheads indicate loss of vessel. (B and C) The number (B) and length (C) of ISVs. * Significantly different from DMSO control ($p < 0.05$). (D) The survival rate of embryos at 24 hpf. The

total number of embryos (B and D) or vessels (C) from three independent experiments is presented in the brackets.

Figure 2. Inhibition of PI3K/Akt signaling impairs vascular development

Embryos were treated with a PI3K inhibitor (LY294002) and an Akt inhibitor (Akt inhibitor) at 5, 10, and 50 μ M concentrations from 9 hpf, and observed at 30 hpf. (A) Representative fluorescent images of vascular structure of embryos treated with each inhibitor (10 μ M). Scale bars indicate 100 μ m. Similar results were obtained from three independent experiments. (B and C) The number (B) and length (C) of ISVs. * Significantly different from DMSO control ($p < 0.05$). N/A, not applicable due to the high lethality. (D) The survival rate of embryos at 24 hpf. The total number of embryos (B and D) or vessels (C) from three independent experiments is presented in the brackets.

Figure 3. Inhibition of PI3K/Akt/mTOR signaling interacts with effects of glycine on vascular development

(A, B and C) Embryos were exposed to glycine (0 and 10 mM) from 3 hpf, treated with or without LY294002, Akt inhibitor, rapamycin, everolimus and KU0063794 (10 μ M) from 9 hpf, and observed at 28 hpf. Control embryos were treated with 0.1% DMSO. (D, E and F) Embryos were exposed to

glycine (0 and 400 mM) from 3 hpf, treated with or without LY294002, Akt inhibitor, rapamycin, everolimus and KU0063794 (10 μ M) from 9 hpf, and observed at 30 hpf. (A, B, D and E) The number (A and D) and length (B and E) of ISVs. * Significantly different from a glycine (0 mM)-DMSO group ($p < 0.05$). (C and F) The survival rate of embryos at 24 hpf. The total number of embryos (A, C, D and F) or vessels (B and E) from three independent experiments is presented in the brackets.

Figure 4. Combination of glycine and an mTOR inhibitor affects expression of *VEGF* and *NOS* genes.

Embryos were exposed to glycine from 3 hpf, and treated with or without KU0063794 (10 μ M) from 9 hpf. The expression of *vegfaa* and *nos2a* genes in whole embryos at 30 hpf was semi-quantified using RT-PCR. (A) Glycine (0 and 10 mM). (B) Glycine (10 mM) with added KU0063794 (10 μ M). (C) Glycine (0 and 400 mM). (D) Glycine (400 mM) with added KU0063794 (10 μ M). (Upper portion of each panel) PCR product images of five samples obtained from three independent experiments. *elfa* was used as an internal control. (Lower portion of each panel) Expression levels were normalized to those of *elfa*. Data are presented as a ratio relative to each control group: a glycine (0 mM) group (A and C), a glycine (10 mM)-DMSO group (B) or a glycine (400 mM)-DMSO group (D). The mean $\pm$ SD (n = 5) was calculated from three independent experiments. * Significantly different from each

control group ($p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4