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Rapamycin treatment causes developmental delay, pigmentation defects, and gastrointestinal malformation on *Xenopus* embryogenesis

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ABSTRACT

Rapamycin is a drug working as an inhibitor of the TOR (target of rapamycin) signaling pathway and influences various life phenomena such as cell growth, proliferation, and life span extension in eukaryote. However, the extent to which rapamycin controls early developmental events of amphibians remains to be understood. Here we report an examination of rapamycin effects during *Xenopus* early development, followed by a confirmation of suppression of TOR downstream kinase S6K by rapamycin treatment. First, we found that developmental speed was declined in dose-dependent manner of rapamycin. Second, black pigment spots located at dorsal and lateral skin in tadpoles were reduced by rapamycin treatment. Moreover, in tadpole stages severe gastrointestinal malformations were observed in rapamycin-treated embryos. Taken together with these results, we conclude that treatment of the drug rapamycin causes enormous influences on early developmental period.

1. Introduction

The target of rapamycin, TOR, is an essential serine/threonine kinase and functions in two distinct multiprotein complexes called as TOR complex 1 (TORC1) and 2 (TORC2). The structure and function of these complexes have been conserved from yeast to vertebrates. As easily imagine from its name, rapamycin binds to TOR kinase and inhibits TORC1 activity although TORC2 is principally insensitive [1].

Rapamycin is originally developed as an antifungal agent. However, this usage was abandoned when it was discovered that it had potent immunosuppressive and antiproliferative properties in human cells. Since then, various roles of rapamycin have been reported. In budding yeast, rapamycin treatment causes a dramatic down-regulation of cellular anabolic processes and correspondingly an up-regulation of catabolic and growth inhibitory processes [1]. Most cells treated with rapamycin abruptly arrest growth and enter a G_0-like state. In mouse, regular consumption of moderate amounts of rapamycin since 600 days of age can extend lifespan; on the basis of age at 90% mortality, it leads to an increase of approximately 10% [2]. In addition, the FKBP–FRB binding assay using rapamycin has been very common tool in cell biology since first reported [3], and it will soon be adopted in other fields such as medical research and developmental biology. However, regarding rapamycin effects for early developmental stage, much remains to be understood although it should be required for the safety use and might be important for thinking the risk during pregnancy and the effect on natural environment. For the investigation to know the effect of rapamycin on early development of vertebrate, aquatic experimental animals must be the best candidates. Interestingly, it is already reported that, in zebrafish, rapamycin treated embryos have mild developmental delay up to 72 hpf (hours post-fertilization) and severe effects on digestive tract development [4]. In this study, we sought to determine the effects of rapamycin during early development of amphibians and found that, in addition to same effects in the case of zebrafish, pigmentation was reduced by rapamycin treatments.

2. Materials and methods

2.1. Embryology and histology

Xenopus laevis eggs were obtained from females injected with 400 units of human chorionic gonadotropin (HCG, Fuji Pharma Co., Japan), fertilized in vitro with minced testis, and then cultured in 0.1× Steinberg's solution (SS). 1× SS contains 58 mM NaCl,

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0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 100 mg/L kanamycin sulfate, and 5 mM Tris–HCl (pH 7.4). Frog embryos were always cultured at 22 °C. The jelly coats were removed with thioglycolic acid solution (1% of thioglycolic acid in 0.1× SS, pH 8.0). Embryos were fixed with PBSFA for 2 h, dehydrated with ethanol, embedded in paraffin wax, sectioned at 8 μm, and stained with hematoxylin and eosin (HE).

2.2. Rapamycin treatment

25 mg of rapamycin (R-5000, LC Laboratories, Massachusetts, USA) was dissolved in 1250 μl of DMSO, and each 20 μl of aliquot (22 mM) was stored at −20 °C. For using, aliquot was diluted with 0.1× SS into proper concentration. Rapamycin (80 μM), which is maximum concentration used in this study, includes 0.36% of DMSO. Note: We confirmed that 0.5% of DMSO was not toxic for development. Embryos were exposed to rapamycin solutions from 2-cell stage to the stages designed for observations.

2.3. mRNA synthesis and microinjection

To generate synthetic mRNA in vitro, pCS2-*dn-Rheb* was linearized with NotI and transcribed with SP6 RNA polymerase by mMESSAGEmMACHINE kit (Ambion, USA). *dn-Rheb* mRNA microinjection into *Xenopus* embryo was performed at 2-cell stage.

2.4. Western blot

Cell lysates were prepared in Phospho Stay Solution (Novagen, USA), and lipids were removed by an equal volume of trichloroethylene (Wako, Japan). Proteins were separated by 5–20% gradient SDS–PAGE gels. Western blots were performed using polyclonal rabbit antibodies against S6K (1:250; CHEMICON, California, USA) and phospho-S6K (1:2000; GeneTex, California, USA).

2.5. RT-PCR

The following primers were used for RT-PCR.

TOR: 5′-CACAGGCTCTAATTTACCC-3′ and 5′-GGTCTCTACCATATG CCT-3′

raptor: 5′-AAGGGGAGAAACTGGACTACTTCCA-3′ and 5′-TTT CATCTCTCGGTCTGTATCC-3′

EF1α: 5′-CCTGAACCACCCAGGCCAGATTGGTG-3′ and 5′-GAGGG TAGTCAGAGAAGCTCTCCACG-3′

Chordin: 5′-CCTCCAATCCAAGACTCCAGCAG-3′ and 5′-GGAGGA GGAGGAGCTTTGGGACAAG-3′

Noggin: 5′-AGTTGCAGATGTGGCTCT-3′ and 5′-AGTCCAAGAGTCT CAGCA-3′

The annealing temperatures were 55 °C, and 25 cycles for *EF1α* and 30 cycles for *TOR*, *raptor*, *Chordin*, and *Noggin* were performed for amplifications.

3. Results and discussion

3.1. TOR and raptor are expressed in all stages of embryos

The TOR protein is conserved from yeast to vertebrate and forms two distinct physical and functional complexes, termed TORC1, which is sensitive to rapamycin, and TORC2, which is insensitive [1]. This inactivation of TORC1 follows the formation of a ternary complex between TOR, raptor, rapamycin, and FKBP12 (Fig. 1B) [5]. FKBP12, which was also called as FK506-binding protein, is expressed in all stages of early development in *Xenopus* [6], meaning that rapamycin does not really require addition of FKBP12 in order to block TORC1 activity. In fact, microinjection of *FKBP12* mRNA did not increase rapamycin activity in our preliminary

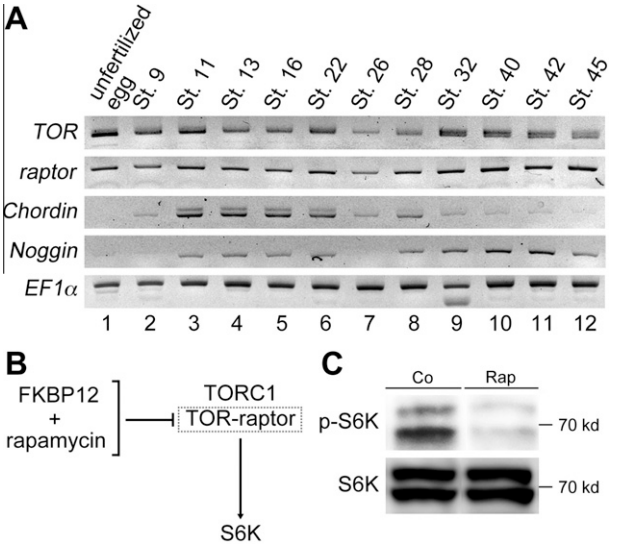


Fig. 1. Inhibition of *Xenopus* TOR activity by rapamycin treatment. (A) RT-PCR analyses for *TOR* and *raptor* mRNA expressions from unfertilized egg to larva stage. Both TORC1 components are consecutively expressed in *Xenopus* embryo. (B) Generally conserved model of TORC1 inhibition by rapamycin. Structure of the FKBP12-rapamycin complex interacts with TORC1 in which TOR and raptor are major components. S6 kinase (S6K) is the major output signal activated by TORC1. (C) Western blot analysis of phospho-S6 kinase (p-S6K) in rapamycin-treated embryos. Embryos were treated at 2-cell stage with 80 μM of rapamycin, and extract was collected at stage 42.

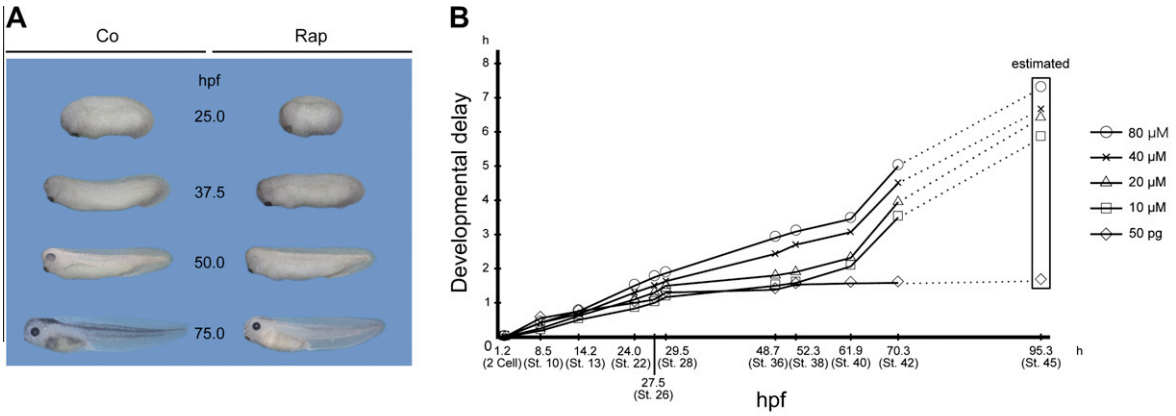
experiments (data not shown). We first analyzed mRNA expressions of *TOR* and *raptor* by RT-PCR (Fig. 1A). In *Xenopus* embryos, zygotic gene transcription is activated at mid-blastula transition (MBT), which is around stage 8.5. Compared to the expression patterns of famous zygotically-expressed genes *Chordin* and *Noggin*, both *TOR* and *raptor* were clearly determined as maternally-expressed genes and continuously expressed in late stages as same as *FKBP12* (Fig. 1A).

3.2. Rapamycin treatment on Xenopus can inhibit TORC1 activity

Ribosomal protein p70 S6 kinase (S6K) is a serine/threonine kinase and a well-established target of TORC1 [7]. We analyzed the effect of rapamycin on S6K phosphorylation targeted by TORC1 by western blot of 3 dpf (days post-fertilization) embryo extracts (Fig. 1C). Using phosphorylated form-specific antibody, we corroborated a substantial decrease in response to rapamycin (Fig. 1C), demonstrating that rapamycin treatment is effective to downregulate TORC1 activity. This reaction interval is somewhat long, but, in zebrafish, which is with almost twice the developmental velocity compared to *Xenopus*, complete inhibition of S6K by rapamycin takes 28 h [4], suggesting that the efficiency of rapamycin treatment for *Xenopus* is equivalent for the case of zebrafish.

3.3. Rapamycin treatment sets back developmental program in dose-dependent manner

To evaluate the effects of rapamycin during development we first used rapamycin treatment (20 μM) on live *Xenopus* embryos beginning at the 2-cell stage up to 3 dpf. Interestingly, developmental speed of embryos treated with rapamycin was clearly delayed, but not arrested (Fig. 2A). Particularly, in terms of optic vesicle formation, distinct difference of developmental speed was observed between control and rapamycin-treated embryos (Fig. 2A). Then, using different concentrations of rapamycin, we tried to measure the relative effectiveness of rapamycin on developmental speed compared to control embryos (Fig. 2B). At 8.5 hpf



C

Averaged delayed time of rapamycin-treated and *dn Rheb* mRNA injected embryo

treatment		hpf of control embryo										
		1.2 (2-cell)	8.5 (St. 10)	14.2 (St. 13)	24 (St. 22)	27.5 (St. 26)	29.5 (St. 28)	48.7 (St. 36)	52.3 (St. 38)	61.9 (St. 40)	70.3 (St. 42)	95.3 (St. 45)
rapamycin	10 μM	0.0	0.2	0.5	0.8	1.0	1.2	1.5	1.6	2.1	3.5	5.8
	20 μM	0.0	0.3	0.6	1.1	1.3	1.5	1.8	1.9	2.3	4.0	6.4
	40 μM	0.0	0.4	0.7	1.3	1.5	1.6	2.4	2.7	3.1	4.5	6.7
	80 μM	0.0	0.4	0.8	1.5	1.8	1.9	2.9	3.1	3.5	5.0	7.3
dn Rheb mRNA	50 pg	0.0	0.6	0.8	1.0	1.1	1.3	1.4	1.5	1.6	1.6	1.7

Fig. 2. The effects of TOR signal by rapamycin treatment and *dn-Rheb* mRNA on developmental speed. (A) Simple comparison of control and rapamycin-treated embryos at same time-course. In embryos treated with rapamycin (20 μM) from 2-cell stage, developmental speed was clearly delayed (100%, $n = 15$). (B) Time-course of developmental delay following rapamycin treatment. In embryos exposed to each test solution (10, 20, 40, and 80 μM), developmental delays were dose-dependently increased (100%, $n = 15$, 100%, $n = 15$, 100%, $n = 15$, and 100%, $n = 15$). Same effect was observed in *dn-Rheb* mRNA (50 pg) injected embryo (100%, $n = 15$). Refer to the scores before stage 42, the estimated delayed times for stage 45 (95.3 hpf in control embryo) were calculated because structural differences induced by rapamycin made collect staging difficult at this stage. Each stage was determined as follow. Stage 10 was determined when blastopore pigmentation is first detected at the dorsal side. Stage 13 when the blastopore was completely closed. Stage 22 when the neural fold was perfectly closed. Stage 26 when dorsal axis was straighten, and primary melanocyte and optic vesicle bump were observed. Stage 28 when fin structure was first observed. Stage 36 when eyes and cement gland were observed clearly and darkly. Stage 38 when fin region was expanded to 50% of trunk-tail. Stage 40 when boundary between posterior yolk and fin was vertically located. Stage 42 when a part of ventral fin was clearly appeared anterior to anus regions. Stage 45 when coiled-coil structure was observed in gut region. (C) Table for B. Rapamycin treatments were performed after 2-cell stage before control embryos reached scheduled stage. *dn-Rheb* mRNA was microinjected at 2-cell stage. Every 15 min we counted number of embryos that already reached scheduled stage. Using these scores, each averaged arrival time for scheduled stages was calculated.

in our experimental condition, 50% of control embryo reached stage 10. On this timing we found that average 0.4 h of delay was observed in embryos treated with 80 μM (Fig. 2B and C). In treatment with 10 μM of rapamycin, obvious difference was detected at 24.0 hpf, which means stage 22; average 0.8 h of delay was turned up in treated (Fig. 2B and C). In all cases time-lag between control and treated embryos were almost directly proportional to incubation period, and in the case of 80 μM it expanded to 5.0 h in stage 42, meaning that it took average 70.3 hpf to reach stage 42 in control embryos and 75.3 hpf (93.4% on developmental speed) in treated (Fig. 2B and C). GTP-binding protein Rheb (Ras homolog enriched in brain) is known as a positive regulator of TORC1 [1]. If rapamycin actually works on TORC1, inhibition of Rheb activity is supposed to make same effect of rapamycin treatment, so we also tried to examine whether inhibition of TORC1 activity by dominant negative form of Rheb (dn-Rheb) has same effect as rapamycin treatment. As a result of this experiment, compared to rapamycin treatment, microinjection of *dn-Rheb* mRNA led almost same level of developmental delay until tailbud stage (Fig. 2B and C), meaning that developmental speed of *Xenopus* embryo was surely controlled by TORC1 pathway. Interestingly, after tadpole stage retardative activity was not observed in the case of mRNA injection (Fig. 2B and C). The reason for this difference happened is probably because mRNA can be targeted by endogenous ribonuclease and completely digested until tailbud stage. As reported, some molecules can arrest development; artificial ERK activation by mRNA microinjection of constitutive active mutant of MEK can immediately block cell cleavage [8]. However, rapamycin-dependent type of developmental delay has never been reported in amphibian embryogenesis. Inhibition of TORC1 signaling has been shown to extend lifespan in yeast [9,10], fruit fly [11], *Caenorhabditis elegans* [12], and mice [2]. Moreover, decrease in speed of larval development by inhibition of TORC1 is also reported in studies using zebrafish and *C. elegans* [4,13]. Lifespan elongation by inhibition of TORC1 may be considered of value from the beginning of life.

3.4. Rapamycin treatment inhibits pigmentation without a decrease in number of melanocytes

In addition to time-course changes we reached a conviction that some structural differences were also occurred in rapamycin-treated embryos. However, in order to avoid regarding time delay-dependent difference as structural change induced by rapamycin, time-course of developmental delay caused by rapamycin should be always taken into consideration in phenotypic evaluations. Therefore, we always referred to table (Fig. 2C) to fix on reasonable timing for rapamycin-treated embryos. For instances, on the condition that control embryo reaches stage 42 at 70.3 hpf, we thought that embryos treated with 10, 20, 40, and 80 μM of rapamycin respectively took 73.8 (+3.5), 74.3 (+4.0), 74.8 (+4.5), and 75.3 (+5.0) hpfs and that *dn-Rheb* mRNA (50 pg) injected embryo took 71.9 (+1.6) hpf for reaching stage 42. Moreover, enormous structural differences caused by rapamycin treatment after stage 42 to be described later made it difficult to do correct staging for rapamycin-treated embryos, so we adopted the way of estimated hpf for late stages embryos. For examples, on the condition that control embryo reaches stage 45 at 95.3 hpf, we regarded that embryos

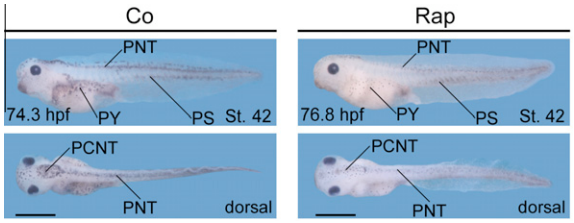


Fig. 3. Pigmentation defects induced by rapamycin treatment. Major pigmentation located at lateral and dorsal side of embryos at stage 42 was obviously detected in control embryos, but weakly in rapamycin-treated (20 μM) embryos (100%, $n = 15$). PCNT, PNT, PY, and PS indicate pigmentation underlain by cephalic neural tube, neural tube, yolk, and somites. Slightly bigger yolk was frequently observed in treated. Scale bars represent 1 mm.

treated with 10, 20, 40, and 80 μM of rapamycin respectively took 101.1 (+5.8), 101.7 (+6.4), 102.0 (+6.7), and 102.6 (+7.3) hpfs and that *dn-Rheb* mRNA (50 pg) injected embryo took 97.0 (+1.7) hpf for reaching stage 45. Using this way, pigmentation defects on the skin was first come under observation (Fig. 3). Usually normal embryos have strong pigmentations on skin underlain by neural tube, yolk, and somites (Fig. 3). On the other hand, these pigmentation, but not retinal pigment epithelium, were much weaker in all rapamycin-treated embryos, and pigmentation underlain by cephalic neural tube was remarkably reduced (Fig. 3). These pigmentation defects were not observed in *dn-Rheb* mRNA injected embryos (Fig. 4C) probably because microinjected mRNA has been already digested when melanocyte formation is started at tailbud stage. In fact, we started rapamycin treatment at stage 20, it actually induced same level of inhibition of pigmentation as normal rapamycin treatment (data not shown). There was not so much of a difference of number of melanocytes between control and treated embryos, indicating that rapamycin may inhibit cell progression but not affect primary differentiation and proliferation of melanocytes. In vertebrate pigment cells found in epidermal skin are derived from neural crest, in contrast pigment cells in the retinal pigmented epithelium originate from the neural epithelium of the optic cup [14]. Therefore, rapamycin treatment may affect somewhere in the step of the segregation of cell lineages derived from neural crest.

3.5. Rapamycin exerts a specific effect on the digestive tract

At stage 42, other structures outside the gastrointestinal tract developed relatively normally despite treatment with rapamycin. In contrast, we found mild phenotypic effects around yolk in rapamycin-treated embryo at stage 42. The size of yolk, which

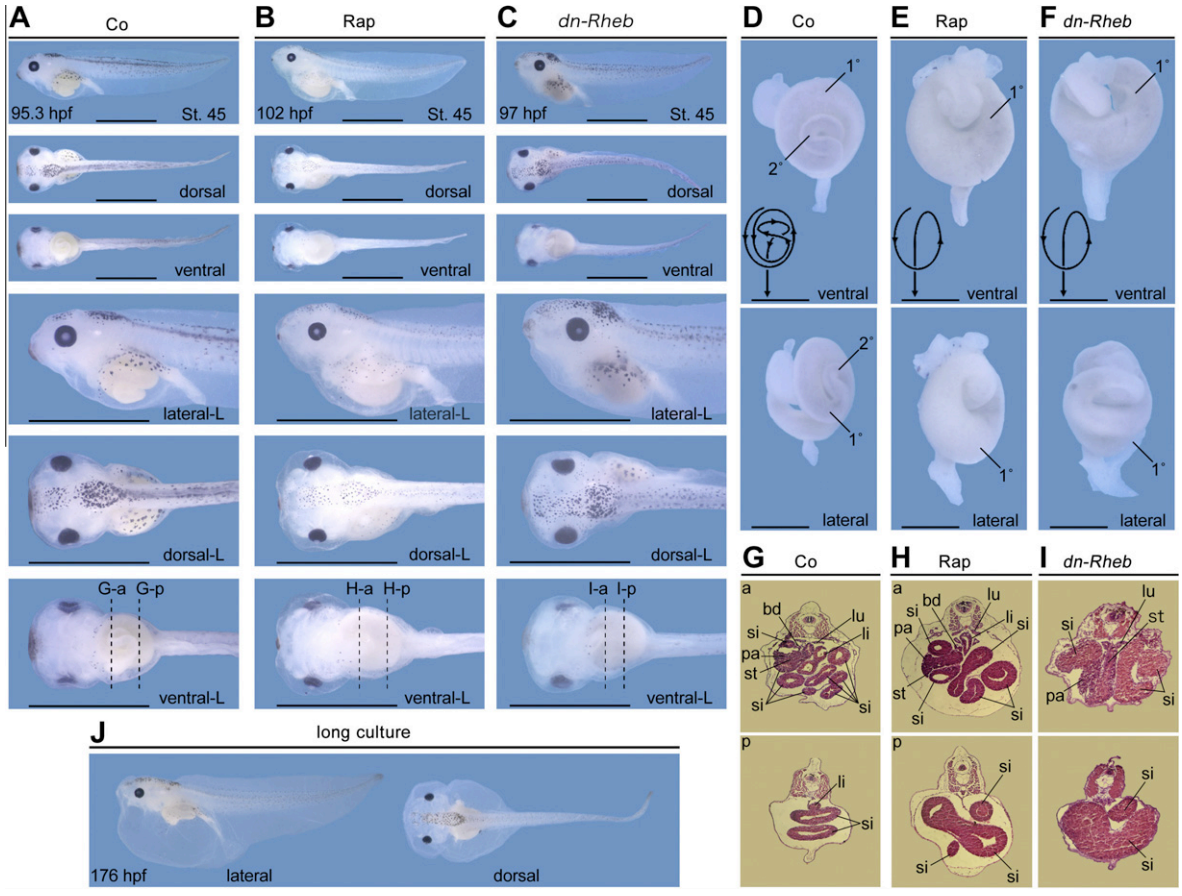


Fig. 4. Gastrointestinal malformation induced by rapamycin treatment and *dn-Rheb* mRNA injection. (A) Control embryo at stage 45 (95.3 hpf) for B and C. (B) Rapamycin-treated embryo at stage 45. Embryos were treated with 40 μM of rapamycin and cultured. Pigmentation defects were stronger than Stage 42. Both size and shape of gut were affected by rapamycin treatment (100%, $n = 15$). (C) *dn-Rheb* mRNA (200 pg) injected embryo at stage 45. Both size and shape of gut were also affected by *dn-Rheb* mRNA injection (100%, $n = 15$), but pigmentations were observed like control. Dotted lines mean sectioned sites for histological analyses shown in G–I. (D–F) Surgically-resected guts from embryos shown in A–C. Upper and under panels respectively indicate ventral and dorsal views. Secondary coiled structure of gut was not detected in rapamycin-treated and *dn-Rheb* mRNA injected embryos. 1°, primary coil; 2°, secondary coil. The diagram of gut structure was shown on the bottom-left corner. (G–I) Histological sections created from embryos shown in A–C. In both rapamycin-treated and *dn-Rheb* mRNA injected embryos, gut formations were disordered and thicker than the case of control embryo. bd, bile duct; li, large intestine; lu, lungs; pa, pancreas; si, small intestine; st, stomach. (J) Long cultured embryos in 20 μM of rapamycin (176 hpf). All embryos were swollen like balloon but survived until 200 hpf maximum. The scale bars of A, B, and C and of D, E, and F represent 2 mm and 0.5 mm, respectively.

is including future gastrointestinal tract, is slightly bigger than normal (Fig. 3). At stage 45, the effect of rapamycin became much more pronounced at several points on treated embryos. Total length of rapamycin-treated embryos was shorter (Fig. 4A and B). Same results were observed in *dn-Rheb* mRNA injected embryos (Fig. 4C). Maturation of melanocytes was clearly observed in control and *dn-Rheb* mRNA injected embryo (Fig. 4A and C), while not in treated (Fig. 4B). Total volume of eye was smaller in both rapamycin treated and *dn-Rheb* mRNA injected embryos, while crystal lens was not properly covered with optic cup only in rapamycin treated (Fig. 4A–C). The gap between skin and inside structure (especially around head region) in treated embryo was wider than in control and *dn-Rheb* mRNA injected (Fig. 4A–C). Particularly, the big effect of rapamycin was detected at the gastrointestinal tract. Normally at this stage the intestine is forming a double-coiled structure, and both external and internal coils have two loops [15]. It was actually found in control embryos (Fig. 4A, D, and G), while rapamycin-treated embryos had single-coiled and much thicker intestine (Fig. 4B, E, and H). Double-coiled structure was not observed even in 176 h cultured embryo with rapamycin (Fig. 4J), indicating that time delay effect is directly attributed to this single-coil structure. Same result was also obtained in the case of *dn-Rheb* mRNA injection (Fig. 4C, F, and I). Intestinal morphogenesis requires a series of changes in the size and architecture of the epithelial cells that line the gut tube. Rapamycin treatment and *dn-Rheb* mRNA injection may give some ramification on presumptive gut region before stage 20, resulting in derangement of timing of ideal communications of each region in gastrointestinal area at later stage. Bile duct, large intestine, and small intestine were not observed in *dn-Rheb* mRNA injected embryo, while all organs were observed in rapamycin-treated embryo same as control embryo. This difference was derived from that TORC1 inhibition by *dn-Rheb* mRNA injection was occurred earlier than by rapamycin treatment (Fig. 4G, H, and I). Almost similar effect by rapamycin is reported in zebrafish and *C. elegans* [4,16]. TORC1 kinase activity might be required for gastrointestinal formation widely in animal kingdom.

3.6. The effect of rapamycin in early development

In this study, we examined the effect of rapamycin on *Xenopus* early development and found three tangible results such as developmental delay, pigmentation defects, and gastrointestinal malformation caused by rapamycin treatment. Moreover, we tried to culture to know how long rapamycin-treated embryos were survived, resulting that all treated embryos were swollen at later stage and kept having gastrointestinal malformation (Fig. 4J). In the end, all died before 200 hpf (stage 47 in control embryo) probably because nutrient supply was interfered with gastrointestinal malformation. Interestingly, mice deficient for *raptor*, which is indispensable component for TORC1, die early in development [17]. The reason why the effect of rapamycin treatment is different from raptor-knocked out phenotype is probably because rapamycin treatment does not affect oocyte or because complete inhibition of TORC1 activity by rapamycin needs long incubations such as 1 day in fish and 2 days in frogs. If faster-acting drug is discovered, it may induce the phenotype like null mutant of *raptor* in mice. It is not clear how TORC1 is communicating with each developmental program that we reported here. However, our data justify special attention to the effect of very popular drug rapamycin in control of very early lifespan of vertebrates.

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