Transgenic expression of Walleye dermal sarcoma virus *rv-cyclin* gene in zebrafish and its protective effect on liver-tumor development after carcinogen treatment

Huiqing Zhan,[1] Jan Spitsbergen,[2] Wei Qing,[1] Yi Lian Wu,[1] Thomas A. Paul,[3] James W. Casey,[3] Zhiyuan Gong[1]*

[1] Department of Biological Sciences, National University of Singapore, Singapore.

[2] Marine and Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, Oregon, USA.

[3] Department of Microbiology and Immunology, Cornell University, Ithaca, New York, USA.

*Corresponding author.

Dr. Zhiyuan Gong

Department of Biological Sciences, National University of Singapore, Singapore, 117543

Tel.: +65 65162860

Fax: +65 67792486

E-mail address: dbsgzy@nus.edu.sg.

Abstract

A retrovirus homologue gene of cellular *cyclin* D_1, walleye dermal sarcoma virus *rv-cyclin* gene (*orf A* or *rv-cyclin*), was expressed in the livers of zebrafish under the control of *liver fatty-acid binding protein (lfabp)* promoter. To prevent possible fatality caused by over-expression of the oncogene, the GAL4/UAS system was used to maintain the transgenic lines. Thus, both GAL4-activator, *Tg*(*lfabp:GAL4*), and UAS-effector, *Tg*(*UAS*:*rvcyclin*), lines were generated and the *rv-cyclin* gene was activated in the liver after crossing these two lines. Since no obvious neoplasial phenotypes were observed in the double-transgenic line, cancer susceptibility of the transgenic fish expressing *rv-cyclin* was tested by carcinogen treatment. Unexpectedly, transgenic fish expressing *rv-cyclin* gene (rvcyclin+) seemed to be more resistant to the carcinogen than were siblings not expressing this gene (rvcyclin–). Lower incidences of multiple and malignant liver tumors were observed in rvcyclin+ than in rvcyclin– fish, and the liver tumors in the rvcyclin+ group appeared later and less severe. These results suggest that expression of *rv-cyclin* protects the fish liver from carcinogen damage and delays onset of malignancy. This observation—from a transgenic fish model—may be relevant to studies of liver-cancer inhibition and regression.

Keywords

Walleye dermal sarcoma virus *rv-cyclin* gene · transgenic fish · liver neoplasia · tumor regression · carcinogen

Introduction [check the right-hand margin below]

The zebrafish (*Danio rerio*) has been used as a carcinogenesis model for over a decade (Hendricks, 1996; Tsai, 1996). The carcinogenicity of diverse compounds at various stages in Florida wild-type zebrafish has been well documented; when treated at the fry or embryo stage, they display a variety of neoplasms derived from epithelial, mesenchymal, neural and neural-crest tissues (Pliss *et al.*, 1982; Khudoley, 1984; Spitsbergen and Kent, 2003; Mizgireuv *et al.*, 2004). These data demonstrated the ease of using zebrafish to assess carcinogen responses *in vivo* and its capacity for developing a diverse range of cancers that pathologically resemble tumor types in humans (Goessling *et al.*, 2007). From these experiments, it has also been noted that zebrafish have a low incidence of spontaneous cancers and show high rates of tumorigenesis after carcinogen treatment (Stern and Zon, 2003), making them an ideal model for chemical carcinogenesis studies. In addition, because a large number of zebrafish can be easily housed and processed for histology, its statistical power is typically greater than that in mammalian carcinogenesis studies (Stern and Zon, 2003). Moreover, fish tumors are commonly seen in the wild and their histological characteristics resemble those of human tumors (Beckwith *et al*., 2000), suggesting that the genetic mechanisms underlying the pathogenic changes associated with malignancy are conserved between fish and human. A comparison of human and zebrafish genome sequences demonstrates conservation of cell-cycle genes, tumor suppressors, and oncogenes (Amatruda *et al*., 2002). Important genes involved in human cancers, such as *p53*, *c-myc*, and angiogenesis genes, have also been found to play roles in fish tumorigenesis (Patton and Zon, 2001). More recently, Lam *et al* (2006) found significant molecular conservation between human and zebrafish liver tumors by comparative

transcriptome analyses. The conserved expression profiles in zebrafish and human cancers provide a molecular basis for validating the zebrafish model in human-disease studies.

Transgenic technology was first successfully used to model cancer disease in zebrafish by over-expressing the mouse oncogene *c-myc* specifically in lymphoid cells, resulting in T-cell acute lymphoblastic leukemia (Langenau *et al.*, 2003). This work indicates the capability of using transgenic zebrafish as a model for cancer progression and metastases in humans and provides a new platform for drug and genetic screens in identifying modifier genes related to *c-myc*-induced carcinogenesis. Recently, several other transgenic zebrafish lines were developed to model specific human cancers, such as pancreatic tumors, melanoma, B-cell leukemia and liver hyperplasia (Yang *et al.*, 2004; Patton *et al.*, 2005; Her *et al.*, 2006; Sabaawy *et al.*, 2006; Park *et al.*, 2008). All of these oncogene-transgenic zebrafish demonstrated the feasibility of generating models to facilitate our understanding of the cancer-formation process, with potential for identifying new therapeutic targets for various cancers.

Studies of tumor induction by retroviruses and DNA viruses have led to key advances in the understanding of cell proliferation and oncogenesis, and laid the foundation for modern cancer studies. Several classes of oncogenes have been identified by retroviral transduction or proviral insertion, including genes coding for tyrosine kinases (*src* and *erbB*), G proteins (*ras*), serine-threonine kinases (*raf*), growth factors (*sis*), and transcription factors (*myc*) (Vennstrom *et al.*, 1982; Privalsky *et al.*, 1984; Finney and Herrera, 1995). Therefore, studies of retroviral oncogenes could provide important clues about the roles of proto-oncogenes in normal cell proliferation and tumor induction. Walleye dermal sarcoma virus (WDSV) is a retrovirus etiologically associated with a

skin tumor termed walleye dermal sarcoma (WDS) that is endemic in walleye fish (*Stizostedion vitreum*) throughout North America (Bowser *et al*., 1988). In contrast to other tumors induced by animal retroviruses, WDS develops and regresses seasonally. Tumors develop in the fall, increase in size until the spring, and disappear in the summer (Bowser *et al.*, 1988; Quackenbush *et al.*, 2001). Therefore, this retrovirus-associated neoplastic disease in walleyes provides a dynamic model for investigating the mechanisms of oncogenesis and tumor progression.

An open reading frame of WDSV, *orf A*, also termed *rv-cyclin*, encodes a homologue of cellular cyclin D. The functional conservation of rv-cyclin and cyclin D has been proven by the ability of rv-cyclin to complement cell-cycle progression in yeast deficient in G_1 cyclins (LaPierre *et al*., 1998). Moreover, *rv-cyclin* has also been shown to prompt squamous epithelium hyperplasia in transgenic mice (Lairmore *et al*., 2000). These observations imply that *rv-cyclin* plays an important role in inducing cell proliferation. However, previous studies also suggested that *rv-cyclin* has a function in WDS tumor regression. *Rv-cyclin* is expressed at low levels in developing tumors and at high levels in regressing tumors (Quackenbush *et al.*, 1997; LaPierre *et al.*, 1998), indicating that it plays a role in tumor regression. Since cyclins are involved in both tumor formation and cell death, *rv-cyclin* may also play a role in WDS tumor regression by inducing cell apoptosis. Therefore, investigation of the function of *rv-cyclin* may help us to understand the mechanisms of oncogenesis and tumor progression.

However, characterization of the walleye retroviruses and their putative oncogenes has been impeded by the lack of a cell-culture system for viral propagation and the difficulty of working with captive wild fish (Paul *et al*., 2001). The zebrafish provides an alternative

system for this study because of their established genetics and development of transgenic technology. In this study, *rv-cyclin* transgenic zebrafish were developed and the function of this gene was characterized. Considering the possible fatality of *rv-cyclin* to the transgenic host, a GAL4/UAS binary transgenic system was adopted to over-express this gene. Since no increase in spontaneous tumor rate in *rv-cyclin*-transgenic fish was observed, 7,12-dimethylbenz[α]anthracene (DMBA) was used to treat *rv-cyclin*-transgenic fish to examine their cancer susceptibility. Unexpectedly, we found that transgenic fish expressing *rv-cyclin* had significantly lower incidences of malignant liver tumor than control siblings. In addition, liver neoplasia developed later in *rv-cyclin*-transgenic fish than that in non-*rv-cyclin* siblings. These observation suggested that expression of *rv-cyclin* in zebrafish liver has a protective effect on the liver-tumor formation induced by carcinogens.

Materials and Methods

Fish maintenance

Zebrafish were maintained essentially according to the Zebrafish Book (Westerfield, 1995). Embryos were cultured in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.0001% Methylene Blue) in a 28.5ºC incubator until 4–5 days postfertilization (dpf). One-month-old fish were transferred to 10-L containers of the Aquatic Habitats (AHAB) recirculating system (Aquatic Ecosystems Inc., FL, USA). During the spawning period, they were kept under a diurnal cycle of 14 h light and 10 h dark.

Construction of GAL4 and UAS plasmids

For the construction of the GAL4 activator plasmid, the *liver-type fatty acid binding protein* gene (*lfabp*) promoter (2.8 kb) was amplified by PCR based on Her et al. (2003), and digested by *Sal*I/*Bam*HI. The promoter fragment was cloned upstream of the translational start site of GAL4 (Scheer and Campos-Ortega, 1999) to make pLFABP-GAL4. For the UAS effector plasmid, the UASfragment was cut from pBlueScript (KS+)-UAS vector (Scheer and Campos-Ortega, 1999) and inserted into the EGFP vector pEGFP1 (Clontech, CA, USA) to make pUAS-EGFP construct.[delete a space]Oncogene *rv-cyclin* (Lairmore *et al.*, 2000) was used to replace the GFP fragment in pUAS-EGFP to make pUAS-RV-CYCLIN construct.

Microinjection and transgenic fish screening

Linearized DNA at a final concentration of 150–200 ng/μL was injected into cytoplasm of zebrafish embryos at the one-cell stage. Each embryo was injected with 2.3 nL of DNA. After microinjection, the embryos were maintained in egg water in an incubator at 28.5°C. All of the injected embryos were raised to sexual maturity and screened for germline transmission. For detection of transgenic founder fish, genomic DNA from pools of F_1 embryos (24 hpf) was isolated according to Westerfield (1995). PCR was carried out with gene-specific primers with a normal PCR program: 94ºC/5 min; thirty cycles of 94ºC/30 sec, 58ºC (variant depend on the annealing temperature of primers)/30 sec, and 72ºC/1 min; followed with 72ºC/10 min for extension. The F_1 embryos from transgenic founder fish were maintained to adult and positive F_1 fish were identified with genomic DNA from fin clips.

Reverse transcription polymerase chain reaction (RT-PCR)

Total liver RNA was isolated by Trizol (Invitrogen, CA, USA). Qiagen One-Step RT-PCR kits (Qiagen, MD, USA) were used for RT-PCR analysis. The PCR program included reverse transcription at 50ºC for 30 min, initial PCR activation at 95ºC for 15 min, followed by three-step cycling: 94ºC/0.5–1min, 56-62ºC/0.5–1 min, 72ºC/1 min, for twenty-eight to thirty-two cycles (*GAL4*, twenty-eight cycles; *rv-cyclin*, thirty cycles), and a final extension at 72ºC for 10 min.

Whole mount *in situ* hybridization and immunohistochemical staining

Whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled riboprobes was carried out as previously described (Korzh *et al.*, 1998). The embryos were fixed with 4% paraformadehyde (PFA) in phosphate-buffered saline (PBS), hybridized with a

digoxygenin (DIG)-labeled *GAL4* RNA probe at 70°C, followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and by staining with the substrates, NBT (nitro blue tetrazolium) and BCIP (5-bromo, 4-chloro, 3-indolyl phosphate). For immunohistochemical studies, embryos were fixed with 4% PFA/PBS, incubated with GAL4 primary antibody (sc-510, Santa Cruz, CA, USA), followed by incubation with the secondary antibody conjugated with horseradish peroxidase and by staining with diaminobenzidene solution.

DMBA treatment

Homozygous *Tg*(*lfabp:GAL4*) was crossed with heterozygous *Tg(UAS:rvcyclin)*. Three-week-old offspring were separated into six groups evenly for carcinogen and vehicle-control treatments. Triplicate groups of fry were treated with 0.75 ppm (mg/L) of DMBA or dimethyl sulfoxide (DMSO, vehicle) for 24 h. Same groups of fry at 6 weeks old were treated with 1.25 ppm DMBA or DMSO again for 24 h. Treated fish were rinsed three times in fresh water and transferred into new tanks for maintenance. Treated fry were maintained in 2.5-L tanks for another month before they were transferred into 4-L tanks in the AHAB system. About seventy fish was randomly selected from each group to be maintained in each tank.

Histopathological analysis

The whole adult fish from DMBA-treated wild-type and *Tg*(*lfabp:GAL4*;*UAS:rvcyclin*) fish were fixed with Bouin's fixative and embedded with paraffin. Nine-step sagittal sections were performed on each fish for tumor diagnosis, covering most of the organs. Sections were stained routinely with hematoxylin and eosin

(H&E). Each fish was genotyped with genomic DNA from fin clips by PCR. Fisher's exact test was used to assess differences between *rv-cyclin* transgenic fish and non-*rvcyclin* siblings.

Results

Generation of *Tg(lfabp:GAL4)* and *Tg(UAS:rvcyclin)* transgenic lines

Of 104 founder fish developed from pLFABP-GAL4-injected embryos, five produced offspring carrying the LFABP-GAL4 fragment in the genome. From seventy-four pUAS-RVCYCLIN-injected founder fish, two *Tg*(*UAS:rvcyclin*) lines (lines 40 and 41) were identified by genomic DNA screening.

Expression of *GAL4* mRNA in *Tg*(*lfabp:GAL4*) was detected in embryos by whole-mount *in situ* hybridization using a DIG-labeled *GAL4* anti-sense RNA probe. In all of the five *Tg*(*lfabp:GAL4*) lines, *GAL4* mRNA was detected specifically in the liver (Fig. 1A). However, expression levels varied between the different activator lines. Among the five activator lines, line 8 showed the strongest expression and was selected for further experiments. Liver-specific expression of GAL4 protein in *Tg*(*lfabp:GAL4*) was also confirmed by whole-mount immunohistochemical staining using GAL4 antibody (Fig 1B). Thus, the *GAL4* transgene was transcribed and translated properly in *Tg*(*lfabp:GAL4*).

Activation of *rv-cyclin* in the livers of adult *Tg(lfabp:GAL4; UAS:rvcyclin)* fish

To test whether *rv-cyclin* effector gene in *Tg*(*UAS:rvcyclin*) could be activated by GAL4, *Tg*(*lfabp:GAL4*) was used to cross with *Tg*(*UAS:rvcyclin*). Liver RNA from

Tg(*UAS:rvcyclin*) and *Tg*(*lfabp:GAL4*; *UAS:rvcyclin*) was used to analyze *rv-cyclin* gene expression. A low level of probably leaky expression of *rv-cyclin* in *Tg*(*UAS:rvcyclin*) was detected by RT-PCR (Lanes 1–3 in Fig. 2). In comparison, significantly higher expression of *rv-cyclin* was detected in the liver of adult *Tg*(*lfabp:GAL4*; *UAS:rvcyclin*) (Lanes 4–6 in Fig. 2). Thus, *rv-cyclin* in *Tg*(*UAS:rvcyclin*) could be activated by the GAL4 protein expressed in *Tg(lfabp:GAL4)*. In the two *Tg*(*UAS:rvcyclin*) lines, line 41 showed higher expression level, therefore we focused on this line in subsequent experiments.

Tumorigenesis in *rv-cyclin* transgenic zebrafish after carcinogen treatment

To examine the effect of *rv-cyclin* on the transgenic zebrafish, homozygous *Tg*(*lfabp:GAL4*) fish were crossed with heterozygous *Tg*(*UAS:rvcyclin*) fish. In this way, 50% of offspring would carry both *lfabp:GAL4* and *UAS:rvcyclin* (rvcyclin+) and the other half siblings would carry only *lfabp:GAL4* (rvcyclin–). Since no obvious spontaneous tumor could be observed in the offspring up to 1 year old, we next tested whether rvcyclin+ double-transgenic fish were more susceptible to carcinogen treatment. DMBA, a carcinogen known to accelerate liver-tumor formation in zebrafish (Spitsbergen *et al.*, 2000), was used to treat the transgenic fish. Due to the lack of a screening marker to differentiate rvcyclin+ double-transgenic fish from rvcyclin– siblings, rvcyclin+ and rvcyclin– fish were maintained together and treated with DMBA (0.75 ppm at 3 weeks and 1.25 ppm at 5 weeks). The rvcyclin+ fish were identified by PCR from fin-clip genomic DNA after dissection. From the 6th month after exposure, some fish treated with DMBA began to show abnormal phenotypes based on gross observation. At 7 months and 10 months post-treatment, sixty-one treated fish of abnormal phenotype

were collected, and examined by nine-step-section for the presence of neoplasias. Of sixty-one fish examined, forty-one were found to carry the *rv-cyclin* oncogene, whereas only twenty were found to be rvcyclin–. Unexpectedly, of those twenty, all (100%) were found to have neoplasias, whereas only thirty-six rvcyclin+ fish (88%) had neoplasias (Fig. 3A). The lower neoplasial incidence found in rvcyclin+ fish indicates that transgenic fish expressing *rv-cyclin* were more resistant to the carcinogen, suggesting that the *rv-cyclin* oncogene provides protection against neoplasia formation.

DMBA-treated *rv-cyclin* transgenic fish had less-severe liver neoplasia than non-*rvcyclin* siblings

Liver and intestinal neoplasias were most commonly induced by DMBA in our fish. For the liver neoplasia, 51% of rvcyclin+ fish (21/41) and 45% of rvcyclin– (9/20) developed liver neoplasia (Fig. 3A). However, livers in DMBA-treated rvcyclin– fish were more severely affected than those in DMBA-treated rvcyclin+ fish. Severity was classified according to the number of liver neoplasias per fish and incidence of malignancy.. As shown in Fig. 3B, rvcyclin+ fish had a lower incidence of multiple liver neoplasias and a higher incidence of single liver neoplasia. Eight out of twenty rvcyclin– fish (40%) had malignant liver neoplasias, whereas only eleven out of forty-one rvcyclin+ fish (27%) had malignant liver neoplasias (Fig. 3C). Furthermore, the incidence of benign liver neoplasias was higher in rvcyclin+ than in rvcyclin– fish (Fig. 3C), again indicating that the liver neoplasias were less severe in rvcyclin+ than in rvcyclin– fish.

We also compared the distributions of liver-neoplasia type in the rvcyclin+ and rvcyclin– fish. As shown in Fig. 3D, hepatocellular adenoma, a benign liver cancer (Fig.

4A), constituted more than 30% of the neoplasias in rvcyclin+ fish but only 21% in rvcyclin– fish. In rvcyclin– fish, most of liver neoplasias were malignant, including hepatocellular carcinoma (29%) and biliary carcinoma (29%) (Fig. 4B, C). In total, thirty-one liver neoplasias were found in rvcyclin+ fish, eighteen (58%) of which were malignant. In rvcyclin– fish, however, eleven of the fourteen (79%) liver neoplasias were malignant, a significant difference based on Fisher's exact test (p <0.01). This result was unexpected and contradictory to our original hypothesis that the transgenic fish expressing *rv-cyclin* in the liver would be more susceptible to the carcinogen and show more malignant liver neoplasias than rvcyclin– fish. These data further confirm that liver neoplasia in rvcyclin- fish is more severe than in rvcyclin+ fish, suggesting that expression of the oncogene *rv-cyclin* in the liver may protect or compensate for the damage caused by DMBA.

In addition, there was also a high incidence of intestinal neoplasia in DMBA-treated rvcyclin+ fish (Fig. 3A), all of which were malignant in both rvcyclin+ and rvcyclin– fish. To investigate the possible effect of *rv-cyclin* expression on the intestinal and liver neoplasia, we examined the *rv-cyclin* mRNA expression in the intestines of adult *Tg*(*lfabp:GAL4;UAS:rvcyclin*) fish. Weak expression of *rv-cyclin* from UAS:rvcyclin was detected in the intestines, but it was comparable to that of the leaky expression in the *Tg(UAS:rvcyclin)* fish (data not shown). Thus, the contribution of *rv-cyclin* expression to intestinal neoplasia was not obvious and there was also little difference in neoplasial incidence between rvcyclin+ and rvcyclin–.

Liver neoplasia developed later in DMBA-treated rvcyclin+ transgenic fish than that in rvcyclin– siblings

Since cancer occurrence and growth may be related to aging (Hasty and Vijg, 2002), we also investigated whether the liver-cancer incidence and severity could be affected by the age of fish. Fish samples were collected at 7 and 10 months after treatment and kept in two groups according to the sampling time.

Liver neoplasial incidence at these two time points is shown in Fig. 5A. At 7 months post-treatment, thirty-four fish of abnormal phenotype were sampled, of which nineteen were identified as rvcyclin+ and fifteen were rvcyclin–. Of the rvcyclin– fish, 53% had liver neoplasias whereas only 32% of rvcyclin+ fish had liver neoplasias. At 10 months post-treatment, twenty-seven abnormal fish were sampled, twenty-two rvcyclin+ and five rvcyclin–. Of these, 68% of the rvcyclin+ fish and 20% of the rvcyclin– fish, developed liver neoplasias. This observation indicates that liver neoplasias developed earlier in rvcyclin– fish than in rvcyclin+ fish.

Neoplasial severity was also analyzed at the two time points. At 7 months post-treatment (Fig. 5B), the incidence of malignant liver neoplasia in rvcyclin– was significantly higher than that in rvcyclin+ ($p < 0.05$); in fact, all of the liver neoplasias in the rvcyclin– fish were malignant. In contrast, at 10 months post-treatment, the incidence of malignancy with rvcyclin+ was higher than with rvcyclin–, and all of the liver neoplasias from rvcyclin– fish were benign. These results further confirmed that the malignant liver neoplasia appeared earlier in rvcyclin– than in rvcyclin+.

We also compared the severity of neoplasias in other organs or tissues, such as intestinal and lymphoid cells, but no significant differences were found between rvcyclin+ and rvcyclin– fish (data not shown).

It appears that the expression of oncogene *rv-cyclin* in the liver negatively affected

progression of liver neoplasias. Since rvcyclin– control fish were more sensitive to DMBA in the induction of liver neoplasia at a younger age than were rvcyclin+ fish, the expression of *rv-cyclin* may play a role in protecting the liver from damage caused by DMBA.

More rvcyclin+ transgenic fish survived from the DMBA treatment than rvcyclin– siblings

To further investigate the protective effect of the *rv-cyclin* gene on DMBA-treated transgenic fish, the percentage of rvcyclin+ and rvcyclin– fish in the total number of surviving *Tg*(*lfabp:GAL4;UAS:rvcyclin*) fish was analyzed. All of the surviving DMBA-treated fish were sacrificed and rvcyclin+ fish were identified by PCR. Since heterozygous *Tg*(*UAS:rvcyclin*) fish were used to cross with homozygous *Tg*(*lfabp:GAL4*) fish, the numbers of rvcyclin+ and rvcyclin– were expected to be approximately equal. In the sampled DMSO-treated control group (n=116), rvcyclin+ fish accounted for 49% and rvcyclin– fish accounted for 51% of those that survived (Fig. 6). These similar percentages indicate that the *rv-cyclin* transgene did not affect survival rate without carcinogen treatment. However, in the DMBA-treated group (n=160), 65% of surviving fish were rvcyclin+ and only 35% were rvcyclin– fish (Fig. 6). The significant difference ($p < 0.01$) between DMBA- and DMSO-treated groups suggests that DMBA had more effect on the survival of rvcyclin– fish. This result confirms that rvcyclin+ fish were more resistant to DMBA.

Discussion

In this study, we generated stable GAL4/UAS transgenic zebrafish lines to evaluate the function of *rv-cyclin* and found that expression of *rv-cyclin* in the liver resulted in fewer malignant liver tumors after carcinogen treatment. We hypothesize that *rv-cyclin* protects against damage induced by DMBA. Since neoplasial severity in liver (but not in other organs) showed significant differences between rvcyclin+ and rvcyclin– fish, liver-specific expression of *rv-cyclin* should be the cause of the difference. This effect on liver might decrease mortality induced by carcinogen treatment (Fig. 6). The mechanism of rv-cyclin protection of the liver from tumor formation may be inferred from the following facts.

Firstly, WDSV is a retrovirus associated with seasonal neoplasia development and regression. While the mechanism of regression is not clear, analyses of regressing tumors in the spring have shown a type of cell death morphologically consistent with apoptosis (Martineau *et al.*, 1990). The *rv-cyclin* gene encodes a cyclin D homolog (LaPierre *et al.*, 1998), and high levels of human cyclin D have been observed in tissue-culture cells undergoing apoptosis (Fukami *et al.*, 1995; Janicke *et al.*, 1996). Since high levels of *rv-cyclin* transcripts have been observed in spring tumors (Poulet *et al.*, 1996; LaPierre *et al.*, 1998), it is possible that it plays a role in tumor regression by inducing apoptosis.

In addition, expression of rv-cyclin protein inhibited cell growth and/or caused cell death in fish and mammalian cells; the first forty-nine N-terminal residues of rv-cyclin were sufficient to cause these effects (Zhang and Martineau, 1999). However, BLAST search found that the N-terminal of rv-cyclin is more conserved to that of cyclin A than that of cyclin D (Zhang and Martineau, 1999). Interestingly, high expression of cyclin A was found to be associated not only with cancer formation (Brechot, 1993) but also with

apoptosis (Hoang *et al.*, 1994; Sozmen *et al.*, 2008). Therefore, rv-cyclin might also arrest the cell cycle and/or cause apoptosis, in addition to causing cell proliferation, as demonstrated by LaPierre *et al.* (1998). In our transgenic fish expressing *rv-cyclin*, it is possible that this gene played stronger roles in cell apoptosis or cell-cycle arrest than in cell proliferation so that the liver in rvcyclin+ fish was more resistant to DMBA-induced oncogenesis. As a result, fewer malignant tumors and lower tumor multiplicity developed in the livers of rvcyclin+ transgenic fish.

It has also been reported that expression of rv-cyclin protein could inhibit eukaryotic promoters, including carp *β-actin* promoter, *WDSV-LTR* (long terminal repeat), CMV immediate early promoter, SV40 early promoter and MMTV full-length LTR (Zhang and Martineau, 1999). Rv-cyclin was found to be able to activate the *WDSV* promoter but to suppress the *SV40* promoter in the same mammalian cells, indicating that rv-cyclin either enhances or inhibits transcription from different promoters in the same cell type (Rovnak and Quackenbush, 2002). In addition, rv-cyclin is also capable of enhancing or inhibiting the same promoter in different cell types (Rovnak and Quackenbush, 2002). These observations demonstrated that the effect of rv-cyclin protein on transcriptional regulation was dependent on both the promoter and the cell type. Therefore, it is possible that the expression of rv-cyclin protein in the liver of our transgenic fish inhibited cancer formation by inhibiting oncogenes and/or activating tumor-suppressor genes.

Moreover, rv-cyclin protein has also been found to be co-localized with RNA polymerase II, cyclin-dependent kinase 8 (Cdk8, the kinase partner of cyclin C), some splicing factors and several transcription factors (Rovnak *et al.*, 2001; Rovnak and Quackenbush, 2002). These factors form a complex required for transcription and mRNA

processing. Due to the structural and functional homology of rv-cyclin with cellular cyclins (LaPierre *et al.*, 1998; Lairmore *et al.*, 2000), rv-cyclin expressed in our *rv-cyclin* transgenic fish might bind with Cdk8 and form a new complex which may change the activity and/or specificity and decrease transcription (Rovnak and Quackenbush, 2002).

The interesting observation from our transgenic fish may be applied to the study of mammalian liver cancer induced by carcinogens. This transgenic model will be a useful tool in the study of cancer inhibition and regression. However, more experiments will be needed to investigate the mechanism of the negative effect of *rv-cyclin* gene on carcinogen-induced liver-tumor formation.

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Fig. 1

Liver-specific expression of GAL4 in *Tg(lfabp:GAL4*) line. (A) The expression of *GAL4* mRNA was detected by whole-mount *in situ* hybridization. (B) GAL4 protein was detected by whole-mount immunohistochemical method. Control: sibling without GAL4 expression. Arrows indicate the liver location. Embryo stage: 4 dpf.

Fig. 2

Activation of *rv-cyclin* in adult livers in *Tg*(*lfabp:GAL4*; *UAS:rvcyclin* line 41) fish as compared with *Tg*(*UAS:rvcyclin*) line 41. Lanes 1–3, three adult fish livers from *Tg*(*UAS:rvcyclin*); Lanes 4–6, three adult fish livers from *Tg*(*lfabp:GAL4*; *UAS:rvcyclin*).

Fig. 3

Comparison of liver tumors in DMBA-treated rvcyclin+ and rvcyclin– fish. (A) Percentages of rvcyclin+ and rvcyclin– developed tumors in DMBA treated *Tg*(*lfabp:GAL4*; *UAS:rvcyclin)* fish. (B) Comparison of single and multiple liver tumor incidence between rvcyclin+ and rvcyclin– fish. (C) Comparison of benign and malignant liver-tumor incidence between rvcyclin+ and rvcyclin– fish. Fish with both malignant and benign tumors were classified as "malignant." (D) Distribution of all types of liver neoplasias in rvcyclin+ and rvcyclin– transgenic fish. Rvcyclin+ transgenic fish show a significantly lower rate of malignant liver tumors (HCC, BC, MC, HB, MM, LMSA), and higher rate of benign tumors (HA, BA, MA) than rvcyclin– fish ($p < 0.01$). HCC, hepatocellular carcinoma; BC, biliary carcinoma/cholangiocarcinoma; MC, mixed carcinoma with hepatocellular and biliary components; HB, hepatoblastoma; LMSA,

leiomyosarcoma; MM, intestinal differentiation in liver tumors with differentiation into malignant enterocytes and malignant smooth muscle; HA, hepatocellular adenoma; BA, biliary adenoma and MA, mixed adenoma with hepatocellular and biliary components.

Fig. 4

Examples of three types of liver tumor. (A) hepatocellular adenoma, (B) hepatocellular carcinoma, and (C) biliary carcinoma. H&E staining. X400. HA, hepatocellular adenoma; N, normal liver tissue.

Fig. 5

Liver neoplasias at 7 and 10 months post-treatment in DMBA-treated rvcyclin+ and rvcyclin– transgenic fish. (A) Comparison of total liver neoplasial incidence at 7 and 10 months post-treatment. (B) Comparison of malignant/benign liver tumor incidence at 7 and 10 months post-treatment.

Fig. 6

Percentages of rvcyclin+ and rvcyclin– fish in total numbers of survived fish after DMSO or DMBA treatment.

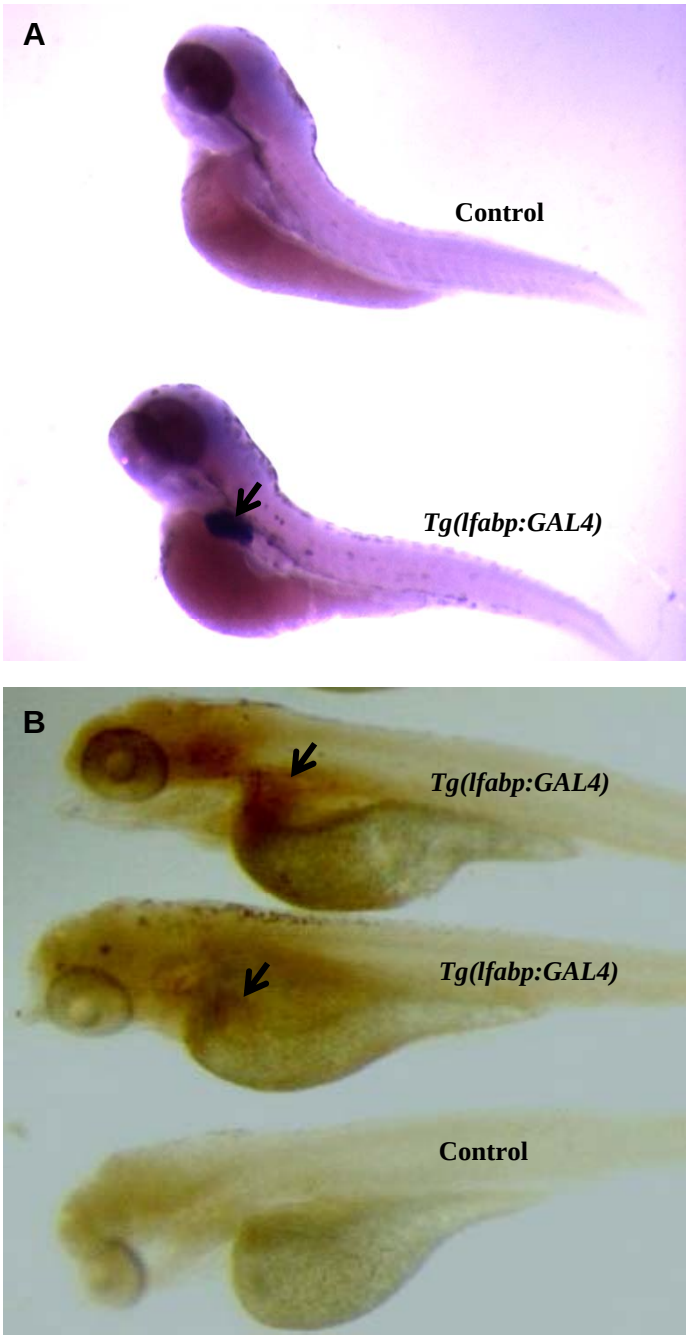


Fig. 1

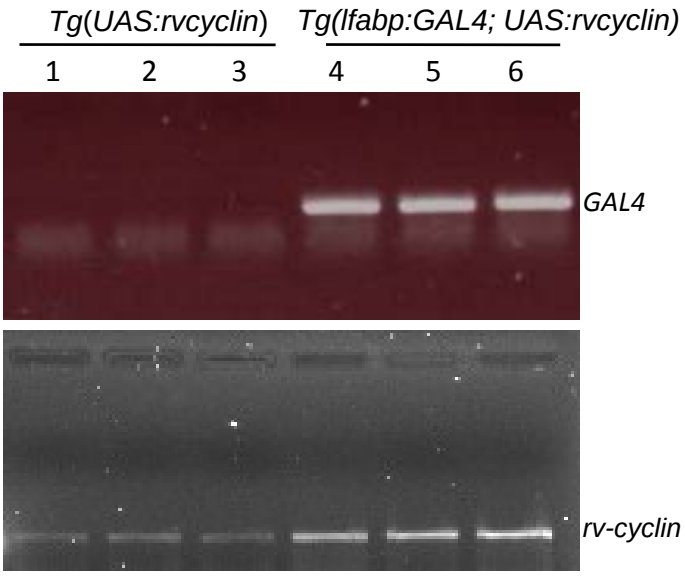

Tg(UAS:rvcyclin)
Tg(lfabp:GAL4; UAS:rvcyclin)
1
2
3
4
5
6
GAL4
rv-cyclin

Fig. 2

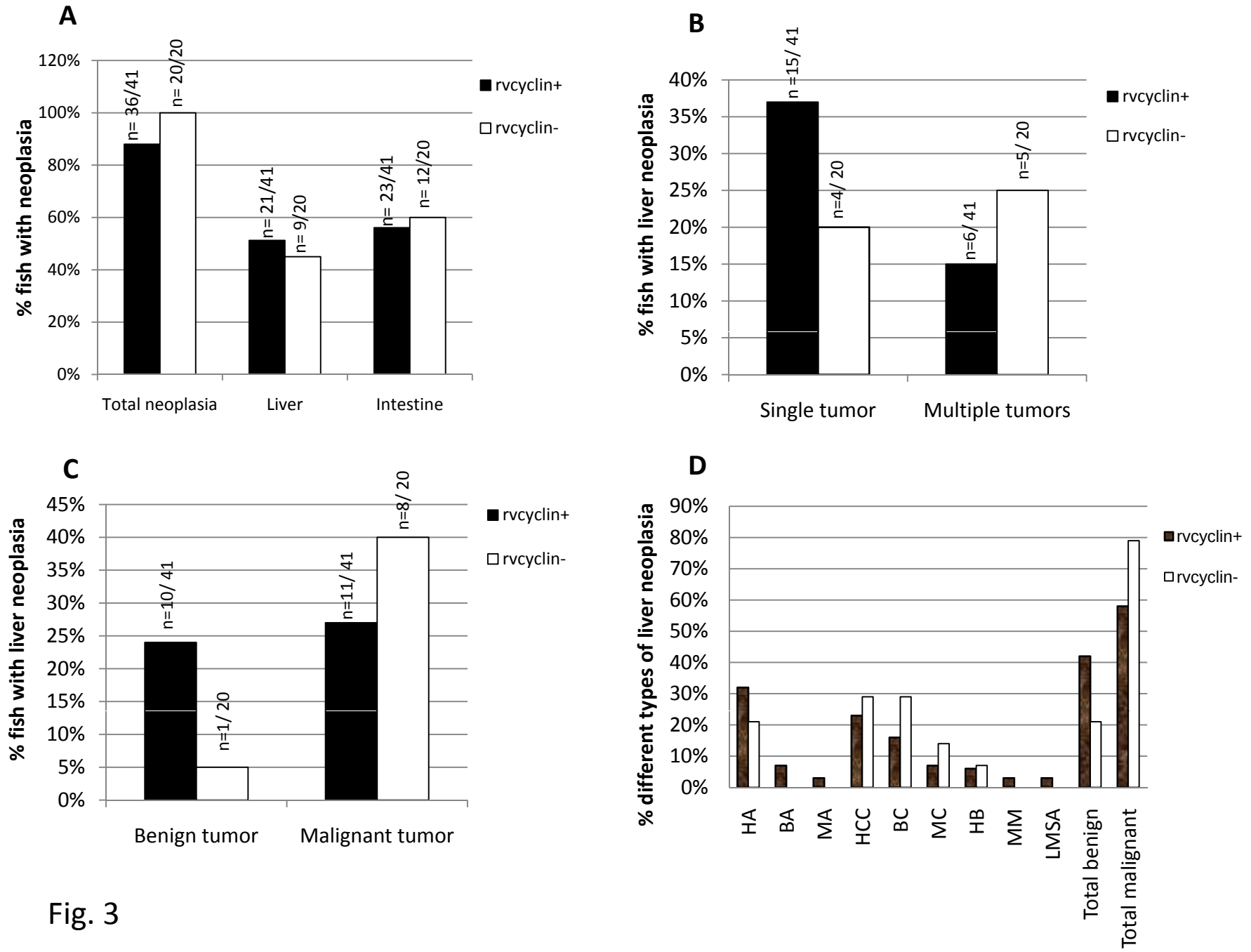

A
% fish with neoplasia
120%
100%
80%
60%
40%
20%
0%
n= 36/41
n= 20/20
n= 21/41
n= 9/20
n= 23/41
n= 12/20
Total neoplasia
Liver
Intestine
rvcyclin+
rvcyclin-
B
% fish with liver neoplasia
40%
35%
30%
25%
20%
15%
10%
5%
0%
n =15/ 41
n=4/ 20
n=6/ 41
n=5/ 20
Single tumor
Multiple tumors
rvcyclin+
rvcyclin-
C
% fish with liver neoplasia
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
n=10/ 41
n=1/ 20
n=11/ 41
n=8/ 20
Benign tumor
Malignant tumor
rvcyclin+
rvcyclin-
D
% different types of liver neoplasia
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
HA
BA
MA
HCC
BC
MC
HB
MM
LMSA
Total benign
Total malignant
rvcyclin+
rvcyclin-

Fig. 3

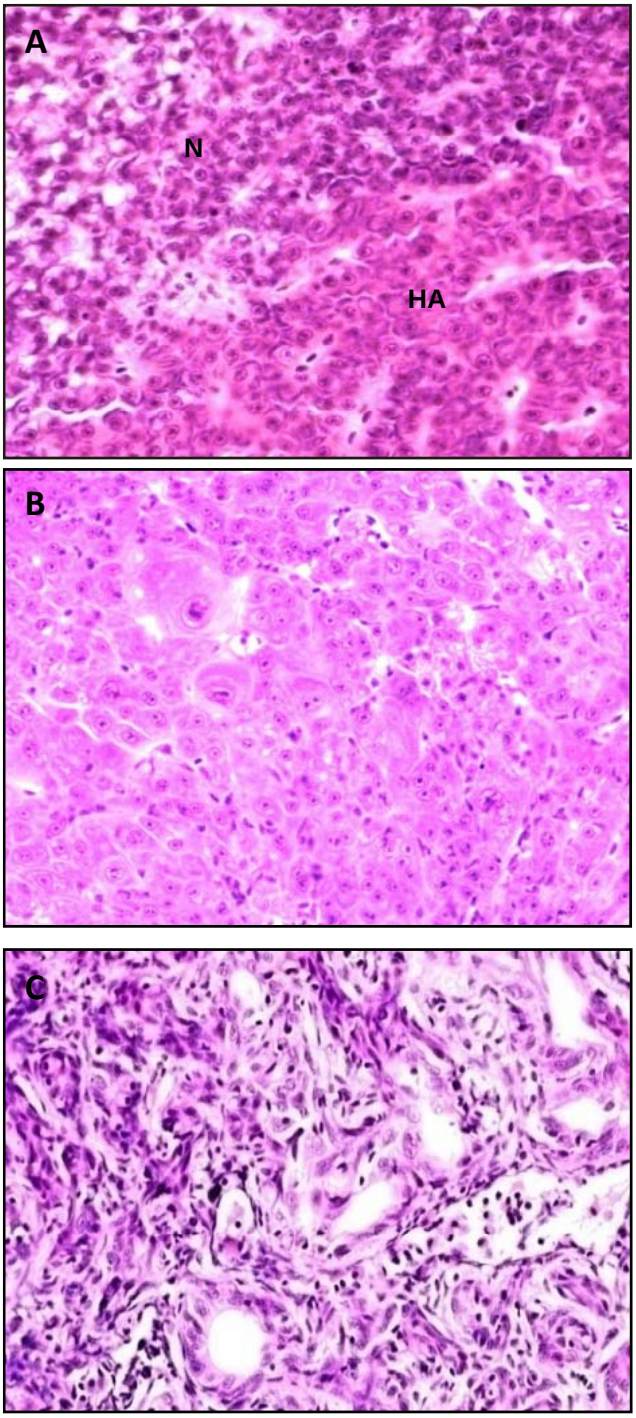
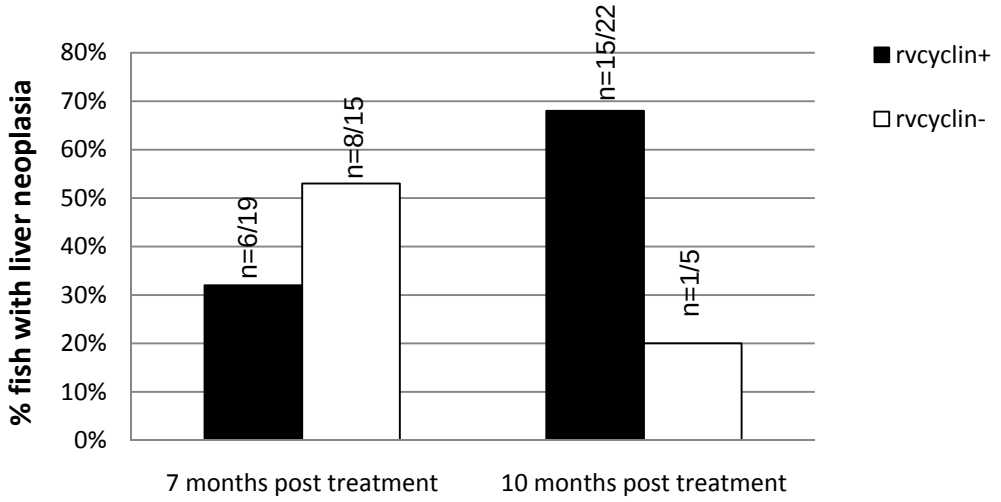


Fig. 4

A


% fish with liver neoplasia
80%
70%
60%
50%
40%
30%
20%
10%
0%
n=6/19
n=8/15
n=15/22
n=1/5
7 months post treatment
10 months post treatment
rvcyclin+
rvcyclin-

B

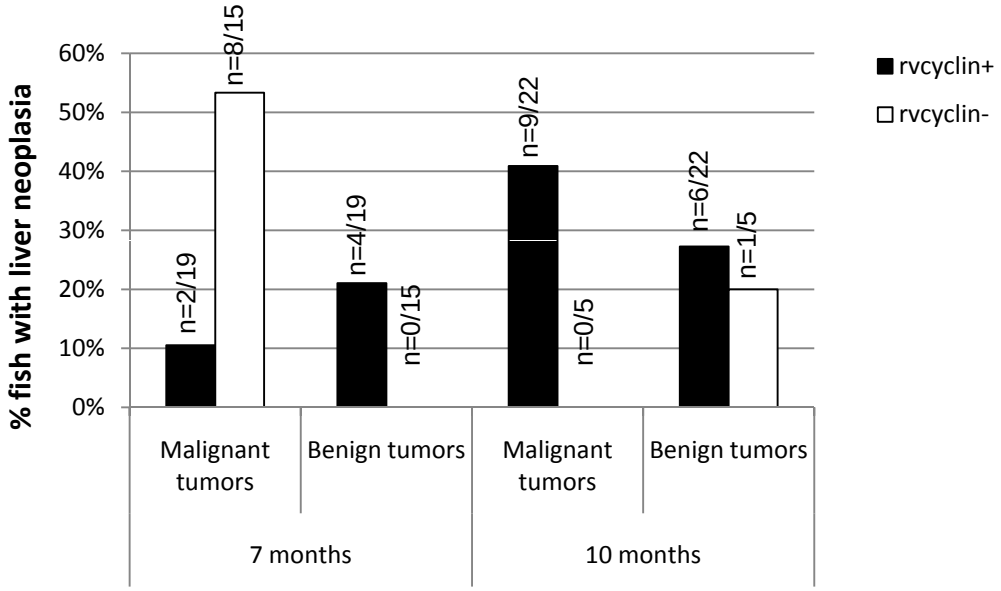

% fish with liver neoplasia
60%
50%
40%
30%
20%
10%
0%
n=2/19
n=8/15
n=4/19
n=0/15
n=9/22
n=0/5
n=6/22
n=1/5
Malignant tumors
Benign tumors
Malignant tumors
Benign tumors
7 months
10 months
rvcyclin+
rvcyclin-

Fig. 5

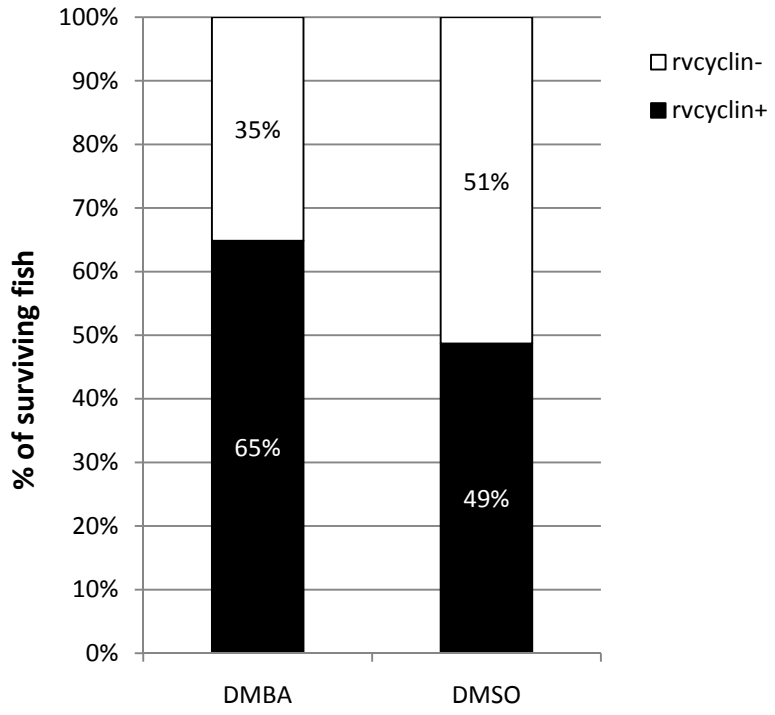

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of surviving fish
35%
65%
51%
49%
DMBA
DMSO
rvcyclin-
rvcyclin+

Fig. 6