doi.org/10.36721/PJPS.2020.33.5.REG.2083-2088.1

Bioluminescence reporter for monitoring G2-phase cell cycle arrest *in vivo*

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Abstract: The bioengineered luciferase reporter has been widely used for monitoring of a variety of molecular events in living cells because of their ability to provide highly sensitive quantitation with broad linearity. In the present study, we made a cyclin A2-luciferase (CYCA-Luc) fusion protein and examined the utility of this optical reporter for monitoring G2-phase cell cycle arrest in living animals. *In vitro* luciferase assay and *in vivo* bioluminescence imaging assay showed that the lithium chloride (LiCl), G2-phase-specific drug, induced G2-phase arrest of cell cycle and increased the activity of this reporter under *in vitro* or *in vivo* conditions, and this reporter can also be potentially used in high-throughput screening efforts aimed at discovering novel anti-cancer drugs that will cause cell cycle arrest at the G2-phase in cultivated cell lines and animal models.

Keywords: Bioluminescence, G2-phase, cell cycle arrest, Cyclin A2-luciferase (CYCA-Luc) fusion, anti-cancer drugs.

INTRODUCTION

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells (Giono, 2002). Currently, there are three known checkpoints: the G1 checkpoint, also known as the restriction or start checkpoint; the G2 checkpoint and the metaphase checkpoint, also known as the spindle checkpoint (Hartwell, 1989; Osborn, 2002). In these checkpoints, G2 cell cycle checkpoints are the final hurdle to prevent a cell from DNA damage going into mitosis, and if DNA damage cannot be repaired, the cell cycle will stop in G2/M phase boundary, leading to certain cell death. Therefore, G2 checkpoint is regarded as important biological target for the treatment of cancer, and G2-phase-target anti-cancer agents are now being developed (Stark, 2004; Kawabe, 2004).

Bioluminescent imaging is a versatile and sensitive tool that is based on detection of light emission from cells or tissues, and it is increasingly being used as a method for rapid, real time monitoring of biological events in living cells (Imamura, 2018). In recent years, the bioengineered luciferase reporter have been widely used in the development of tools for monitoring biological events in living cells (Mori, 2008; Jimenez, 2011). The cell cycle specific protein is well-suited as a molecular marker to reflect cell cycle progression in living cells because it is regulated by cell cycle pathways (Zhang, 2004: Zhang, 2013; Chen, 2013). Cyclin A2 is a cell cycle control protein that plays an important role in cell proliferation and cancer (Dachineni, 2016). In mammalian cells, cyclin A2 levels are low during G1-phase but increase at the G2-phase and it is degraded during prometaphase by proteasomal ubiquitin-dependent proteolysis (Den Elzen, 2001). Based on this principle, in the present study, we have developed a cyclin A2-luciferase (CYCA-Luc) fusion protein for monitoring G2-phase of the cell cycle in living animals. Our results demonstrate that the CYCA-Luc fusion protein reporter system can be used to monitor G2-phase arrest and pharmacological activity of G2-phase-specific drug, lithium chloride (LiCl) in real time *in vitro* and *in vivo*.

MATERIALS AND METHODS

Luciferase Assay System was purchased from Promega (Madison, WI, USA); D-Luciferin was purchased from AAT Bioquest, Inc. (Sunnyvale, CA); Lithium chloride (LiCl) and colchicine were purchased from YTHX Biotechnology Co., Ltd. (Beijing, CN); Lipofectamine 2000 transfection agent was purchased from Invitrogen (Camarillo, USA); BALB/C nude mice, 4-6 weeks of age (15-20g), were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, CN). Rabbit polyclonal antibody against luciferase was purchased from R&D (Minneapolis, USA).

Cell Culture, transfection and tumor xenografts assay

HeLa cells were purchased from the Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HeLa cells are maintained in DMEM medium (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY, USA), 100IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO2. Plasmids transfection and tumor xenografts assays were performed as described previously (Chen, 2013).

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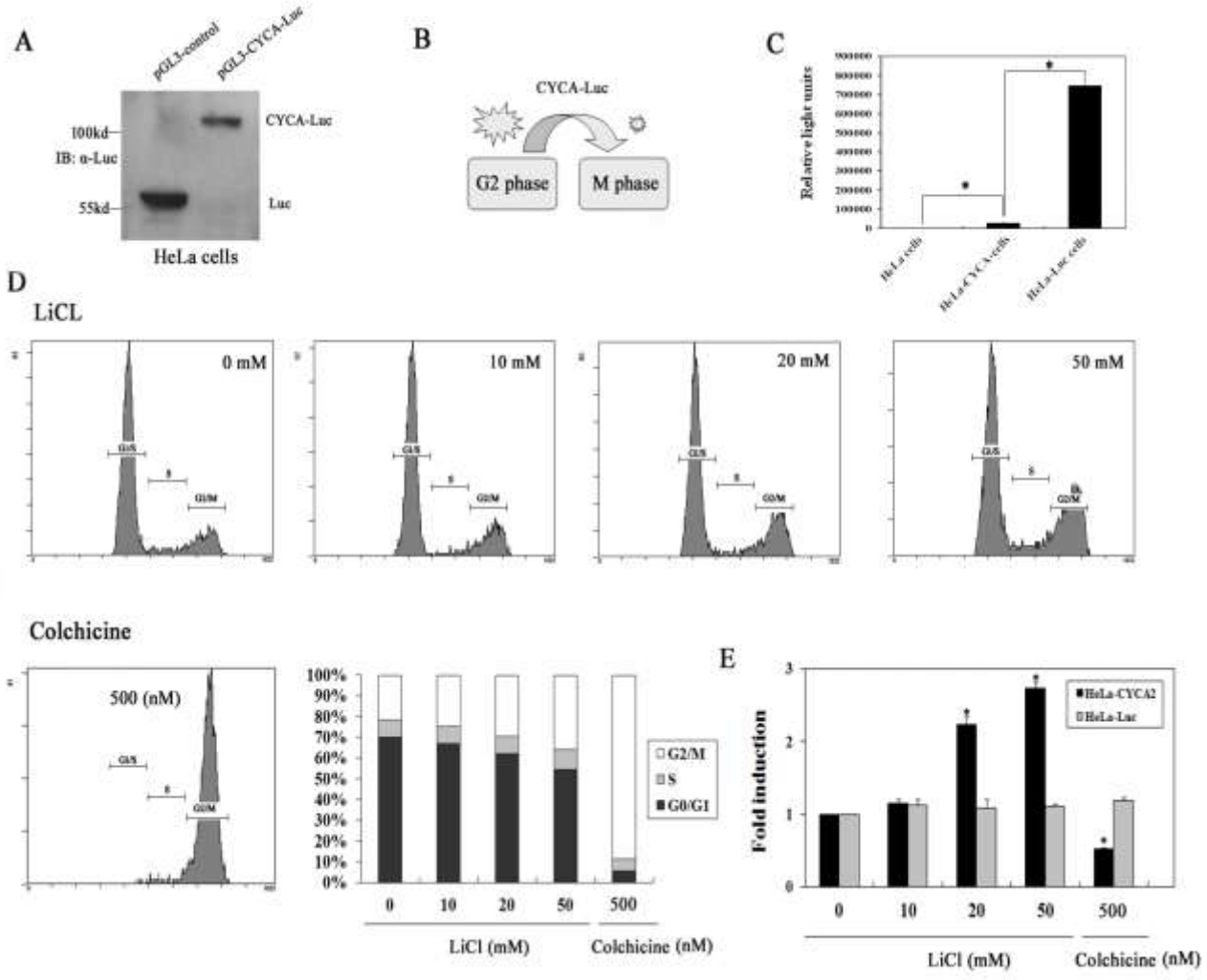

Fig. 1: LiClupregulate CYCA-Luc activity by inducing cell cycle G2-phase arrest.(A) HeLa cells were placed into wells of a 6 well plate and transfected using the Lipofectamine 2000 with equal concentrations of plasmid DNA encoding either pGL3 or pGL3-CYCA-Luc. Transfected cells were cultured for 48h, lysed, and cell extracts were immunoblotted with the indicated antibodies.(B) Schematic diagrams of the G2-phase reporter. (C) HeLa cells, HeLa-CYCA-Luc cells or HeLa-Luc cells were lysed, and cell extracts were assayed for luciferase activity. For normalization of CYCA-Luc activity or Luc, the signal (1μg protein) for control was set to 1.(D) HeLa-CYCA-Luc cells or HeLa-Luc cells were synchronized by growth in media containing LiCl (0, 10, 20 and 50mM) or 500nM colchicine for 48h, and cells were analyzed for DNA content by FACS after propidium iodide staining, or were lysed. (E) Cell extracts were assayed for luciferase activity. For normalization of luciferase or CYCA-Luc activity, the signal for untreated cells was set to 1. This experiment was repeated three times (n=3). Error bars indicate standard error; *, $p<0.05$ compared with PBS.

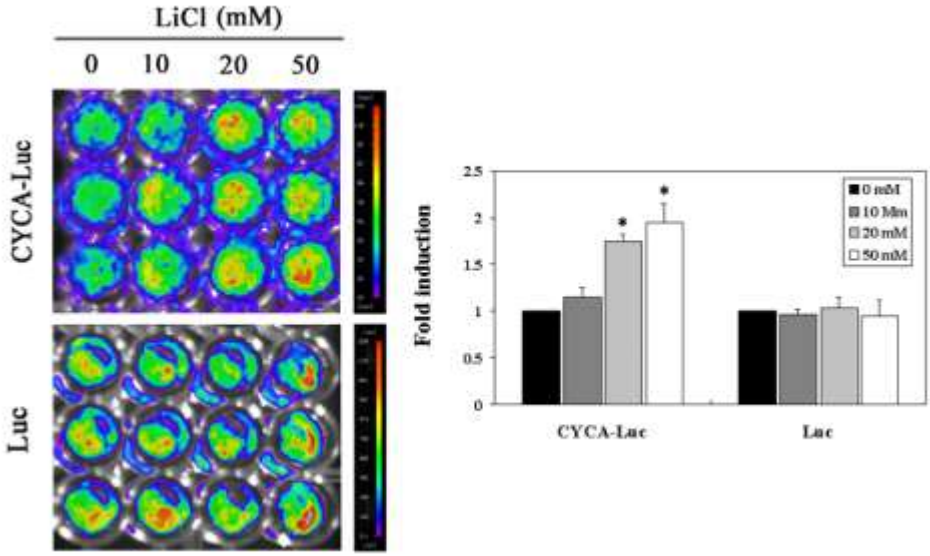

Fig. 2: Induction of CYCA-Luc by G2-phase-specific drug, LiCl *in vitro*. HeLa-CYCA-Luc cells or HeLa-Luc cells were placed into wells of a 96 well plate. Bioluminescence signals were quantified after 48 h treatment with LiCl (0, 10, 20 and 50 mM). Left, cellular images obtained after treatment with LiCl. Right, normalized fold induction of CYCA-Luc or Luc treated with the indicated doses of drugs. For normalization of luciferase or CYCA-Luc activity, the signal for untreated cells was set to 1. Quantitative data represent the mean ± standard error (n = 3 per group).

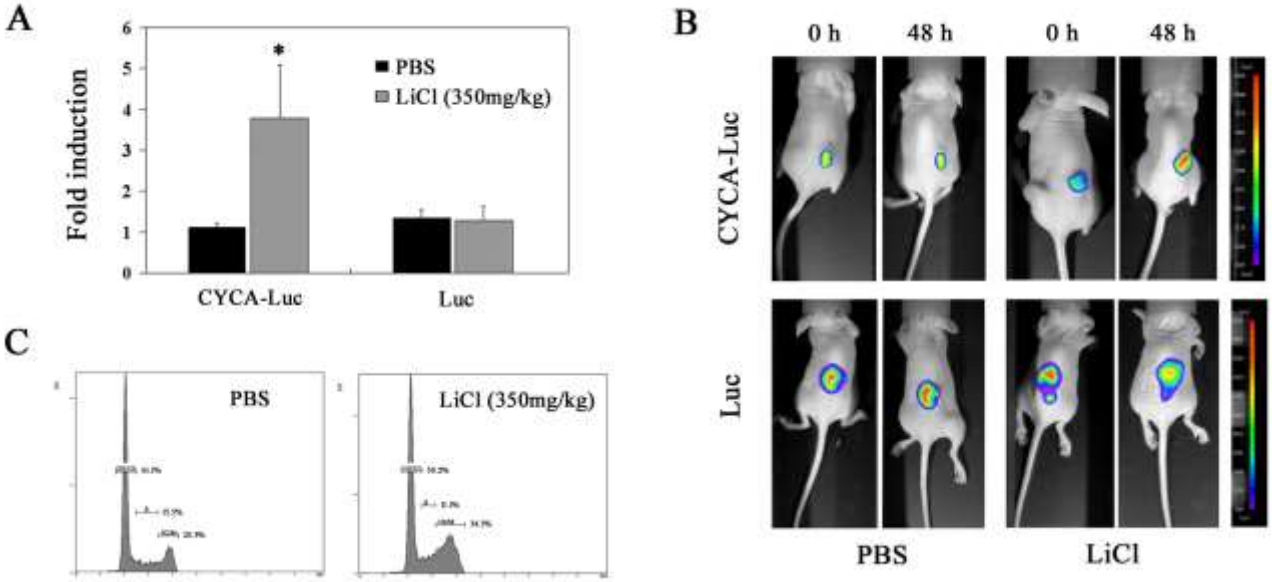

Fig. 3: Monitoring G2-phase-specific drug *in vivo* using bioluminescent imaging.(A, B) 1×107 HeLa-CYCA-Luc cells or HeLa-Luc cells in 0.2 mL PBS were injected subcutaneously into each flank of BALB/C nude mice under anesthesia (isoflurane). Mice were treated with PBS or 350mg/kg LiCl, for 48h, by intraperitoneal injection (n=3 per group). Bioluminescence images were acquired at the 10min time points after intraperitoneal injection of D-Luciferin, and fold induction is expressed as a ratio of post treatment versus pretreatment bioluminescence data from three mice; error bars indicate standard error; *, $p<0.05$ compared with PBS. (C) Cells were isolated from PBS or LiCl-treated tumor tissues and were analyzed for DNA content by FACS after propidium iodide staining.

To make stable cell lines (HeLa-CYCA-Luc cells and HeLa-Luc cells), HeLa cells were co-transfected with 5μg of pGL3-control or pGL3-CYCA-Luc and 0.5μg of empty pcDNA3.1 (Invitrogen). Forty-eight hours later, cells were placed in medium containing G418 (1000μg/mL) to antibacterically select transfected cells to grow.

Western blotting

Proteins from cultured cells were extracted by lysis in Reporter Lysis Buffer (RLB) buffer and quantitated by BCA protein assay. Equal amounts of protein per lane were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies against luciferase. Protein bands were developed using horseradish peroxidase-conjugated secondary antibodies and developed using chemi-luminescence.

Cell cycle analysis

HeLa cells stably expressing CYCA-Luc were blocked by incubation in medium containing LiCl (0, 10, 20, 50mM) or colchicine (500nM) for 48h. Cells were trypsinized, washed twice with Phosphate Buffered Saline (PBS), and fixed in 70% ethanol at 4°C for at least 1h. Fixed cells were centrifuged and resuspended in 50μg/mL RNase and 10μg/mL propidium iodide for DNA staining at 37°C for 30 min. DNA content was measured by flow cytometry (Beckman Coulter, Miami, FL, USA).

In vitro and in vivo bioluminescent imaging

For *in vitro* bioluminescence imaging studies, D-Luciferin was added to tissue culture medium to a final concentration of 150μg/mL. Five minutes later, photons were counted using the Night OWL LB 983 *in vivo* imaging system (Berthold Technologies). Data was analyzed using IndiGO2 software. For *in vivo* studies, at 48h after intraperitoneal administration of 350mg/kg lithium chloride (LiCl), mice were administered 150 mg/kg D-Luciferin, by intraperitoneal injection. The anesthetized mice were placed onto a warmed stage inside the light-tight box. In this study, mice were imaged 10 minutes after D-Luciferin injection to ensure consistent photon flux emitted during the oxidation of the substrate. The CCD deep-cooled camera system was used to visualize tumors, and photon measurement was defined around the tumor area and quantified using IndiGO2 software.

Luciferase assay

Luciferase activity in cell extracts was assessed using the Promega Luciferase Assay System according to the manufacturer instructions (Promega). Washed cells were lysed using the lysis reagent provided in the kit. The Plate-Reading luminometer's injectors added 50μL of the kit Luciferase Assay Reagent providing the D-Luciferin substrate into 96-well plate containing 20μL of cell lysate per well. Photon emission was measured for a period of 10 seconds with a delay time of 2 seconds after injection of the substrate.

STATISTICAL ANALYSIS

Data was expressed as mean ± standard deviation of the mean (SD). Statistics were analyzed using Student's t-test. p values less than 0.05 were considered statistically significant. Statistical analysis was done by SPSS 15.0 and Graph Pad Prism 5.0 software.

RESULTS

CYCA-Luc accumulates in response to G2-phase blockage

The pGL3-CYCA-Luc plasmid encoding full-length cyclin A2 fused in frame to the N-terminus of firefly luciferase was constructed (data not shown). The pGL3 or pGL3-CYCA-Luc vector was transfected into HeLa cells, and the CYCA-Luc fusion protein or luciferase (Luc) protein was detected by immunoblot analysis (fig. 1A). It is well known that cyclin A2 levels are low during G1-phase but increase at the G2-phase (Dachineni, 2016). In this assay, therefore, we examined whether the CYCA-Luc accumulates in response to G2-phase blockage, as showed in fig. 1B. First, we established the HeLa-CYCA-Luc cells, stably expressing CYCA-Luc, and the HeLa-Luc cells, stably expressing Luc, and substantial luciferase activity was detected (fig. 1C). To investigate whether the CYCA-Luc levels increase at G2-phase, the HeLa-CYCA-Luc cells were blocked with different concentration (0, 10, 20 and 50mM) of the lithium chloride (LiCl), arresting cell cycle in the late G2-phase; or with 500 nM colchicine, an M-phase blocker, arresting cell cycle in the M-phase. At 48 h after treatment, the percentage in the G2-phase was increased significantly with the increasing doses of LiCl, while the colchicine treatment for 24 h increased the accumulation of M-phase cells (fig. 1D). As expected, a luciferase assay demonstrated that colchicine down regulated the CYCA-Luc reporter activity, while LiClupregulated its activity in a dose-dependent manner, consistent with cell cycle distribution. In contrast, wild-type luciferase showed no significant change after treatment with LiCl or colchicine (fig. 1E). These data suggested that the CYCA-Luc accumulates in response to G2-phase blockage, and this reporter activities indeed reflect the effect of G2-phase arrest drug, LiCl *in vitro.*

Bioluminescence imaging of G2-phase arrest in vitro

We next asked whether CYCA-Luc bioluminescence activity could be used to monitor the effect of G2-phase-specific drugs in cellulo. It is well known that bioluminescence imaging (BLI) uses light measurements (FLUX) to monitor tumor progression and drug responses, and the FLUX data represents the number of luciferase positive cells in a given region of interest (Tiffen, 2010). HeLa-CYCA-Luc cells derived from culture were prepared and placed in wells of a 96-well plate and after cells were treated with different concentrations of LiCl for

48h, D-Luciferin was added directly to the cell culture medium and the plate was then imaged using the Night OWL LB 983 *in vivo* imaging system. As expected, LiCl caused a dose-dependent increase in luciferase signals; whereas LiCl treatment did not show a conclusive effect on luciferase signals in HeLa-Luc cells (fig. 2).

Bioluminescence imaging of G2-phase arrest in vivo

The experiment above proved that the CYCA-Luc bioluminescence activity could be used to monitor the activity of G2-phase arrest drug, LiCl under *in vitro* conditions. In this assay, we further determined if this reporter can monitor G2-phase arrest under *in vivo* conditions. We first characterized its bioluminescence reaction properties in tumor-bearing nude mice. Nude mice were injected subcutaneously with 1×107 HeLa-CYCA-Luc cells or HeLa-Luc cells. Ten days later, the mice were imaged using the Night OWL LB 983 *in vivo* imaging system after intraperitoneal injection of D-Luciferin (150mg/kg) and acquiring photon counts 5, 10, 15, 20 and 25 minutes respectively, after injection. We observed that bioluminescence signal reached plateau phase at the 10min time point (data not shown); therefore, animals were imaged at a standard point (10 min) after D-Luciferin injection. Next, we examined the effect of LiCl on bioluminescence signals from HeLa-CYCA-Luc tumors or HeLa-Luc tumors. The bioluminescence was detected at 48h after LiCl (350mg/kg) or PBS administration with *in vivo* imaging. As expected, the bioluminescent signal was significantly induced by LiCl *in vivo*, but we did not find significant differences in the PBS treatment group. To confirm that the bioluminescent signal induction is indeed caused by CYCA-Luc activity change, cells were isolated from these tumor tissues and were assessed by flow cytometry. At 48 h after treatment, the percentage in the G2-phase was increased significantly, suggesting that the reporter induction is caused by G2-phase arrest (fig. 3C). These results suggested that the CYCA-Luc fusion protein was suitable for monitoring G2-phase specific drug, LiCl treatment in living animals.

DISCUSSION

Drug screening is a key step in the drug discovery and development process. In recent years, High-throughput screening has become an indispensable tool for the pharmaceutical industry and for biomedical research (Fraser, 2017). However, ideal *in vivo* tumor models for *in vivo* drug screening remain limited. New *in vivo* technologies for screening anti-cancer drugs are urgently needed.

The bioengineered luciferase reporter is being used to develop ultra sensitive tools for *in vivo* imaging of various proteins, thanks to their high detectability and to the availability of highly sensitive bioluminescence instruments (Jimenez, 2011; Zhu, 2017). Many aspects of drug development can be facilitated using bioluminescence reporter proteins as an indicator to discover new targets, identify novel drug candidates, and validate their potency (Chen, 2013). In the present study, we found that a reporter consisting of cyclin A2 fused to luciferase was responsive to G2-phase-specific anti-cancer drug, LiCl in cellulo and *in vivo*. Responsiveness to drug activity was validated using the Night OWL LB 983 imaging system. We demonstrated that the CYCA-Luc reporter can provide a pharmaco-dynamic readout of G2-phase specific anti-cancer drug action, LiCl in animal models.

Lithium chloride (LiCl), which is a specific inhibitor of glycogen synthase kinase-3β, is known to induce cell cycle arrest at the G2-phase and it was as a G2-phase-specific drug used in this study and our expectation is that this CYCA-Luc can reflect its activity for arresting G2-phase (Ha, 2014; Li, 2015). As expected, we observed that LiCl caused CYCA-Luc activity upregulation whether *in vitro* or *in vivo*; whereas colchicine, M-phase-specificity drug, caused CYCA-Luc activity down regulation. Thus, these results demonstrated that this reporter can be used to monitor cell cycle G2-phase arrest, and detect the G2/M phase transition *in vivo* and *in vitro*. Precisely because of this capability, it overcomes the limitations of flow cytometry which cannot distinguish G2 from M-phase, and as a rapid and accurate approach, it can directly reflect G2-specific anti-cancer drug activity through detection CYCA-Luc activity *in vitro*, and not need to undergo cumbersome process as western blot, flow cytometer, etc. More importantly, it is bioengineered luciferase reporter, which can be used to monitor the G2-phase arrest in living animals with noninvasive bioluminescence imaging and therefore has potential applications for evaluating pharmacological activity of G2-phase targeted anticancer drug in real time *in vivo*.

CONCLUSION

This CYCA-Luc reporter can be used to screen for anti-proliferative compounds that may arrest cell cycle in the G2-phase and then trigger apoptosis. Thus, this reporter system provides an ideal tool for high-throughput screening of G2-target anti-cancer and investigating the G2/M transition mechanisms *in vitro* and *in vivo.*

ACKNOWLEDGEMENTS

This project was supported by Science and Technology Project of Heilongjiang Education Department (No. 12531729; 2016-KYYWF-0668), Natural Science Foundation of Guangxi Province of China (No. 2019JJA140432) and National Natural Science Foundation of China (No. 81271628; 31860313).

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