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Suppression of *Ov-grn-1* encoding granulin of *Opisthorchis viverrini* inhibits proliferation of biliary epithelial cells

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Abstract

Multistep processes likely underlie cholangiocarcinogenesis induced by chronic infection with the fish-borne liver fluke, *Opisthorchis viverrini*. One process appears to be cellular proliferation of the host bile duct epithelia driven by excretory-secretory (ES) products of this pathogen. Specifically, the secreted growth factor *Ov*-GRN-1, a liver fluke granulin, is a prominent component of ES and a known driver of hyper-proliferation of cultured human and mouse cells *in vitro*. We show potent hyper-proliferation of human cholangiocytes induced by low nanomolar levels of recombinant *Ov*-GRN-1 and similar growth produced by low microgram concentrations of ES products and soluble lysates of the adult worm. To further explore the influence of *Ov*-GRN-1 on the flukes and the host cells, expression of *Ov-grn-1* was repressed using RNA interference. Expression of *Ov-grn-1* was suppressed by 95% by day 3 and by ~100% by day 7. Co-culture of *Ov-grn-1* suppressed flukes with human cholangiocyte (H-69) or human cholangiocarcinoma (KKU-M214) cell lines retarded cell hyper-proliferation by 25% and 92%, respectively. Intriguingly, flukes in which expression of *Ov-grn-1* was repressed were less viable in culture, suggesting that *Ov*-GRN-1 is an essential growth factor for survival of the adult stage of *O. viverrini* survival, at least *in vitro*. To summarize, specific knock down of *Ov-grn-1* reduced *in*

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vitro survival and capacity of ES products to drive host cell proliferation. These findings may help to contribute to a deeper understanding of liver fluke induced cholangiocarcinogenesis.

Keywords

O. viverrini; cholangiocarcinoma; RNA interference; granulin; liver fluke; cellular proliferation

1. Introduction

The International Agency for Research on Cancer recognizes infection with three helminth parasites as definitive causes of cancer as members of its Group 1 Carcinogens - the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke *Schistosoma haematobium* (Bouvard et al., 2009, de Martel et al., 2012, IARC, 2012). Liver fluke infection is of major public health concern in East Asia and Eastern Europe, where more than 40 million people are infected. *O. viverrini* infection is endemic in Thailand, Laos, Vietnam and Cambodia (Sithithaworn et al., 2012, Sripa et al., 2011). People acquire opisthorchiasis following ingestion of raw cyprinid fishes containing the metacercarial stage of *O. viverrini* (Sripa, 2012). Infected people may not exhibit clinical signs and symptoms; however, 5-10% of patients with chronic infection will progress to hepatobiliary disease including obstructive jaundice, hepatomegaly, cholecystitis, and/or cholangiocarcinoma (CCA) (bile duct cancer) (Smout et al., 2011, Sripa et al., 2012, Sripa et al., 2007). CCA is the eminent type of liver cancer in Thailand and Laos where consumption of uncooked fishes harboring the infectious larvae of this liver fluke is a dietary staple (Mairiang et al., 2012, Sripa and Pairojkul, 2008). Problematically, whereas the strong link between opisthorchiasis and CCA has been known for decades, the incidence of CCA remains high (Mairiang et al., 2012, Sripa et al., 2007). In resource limited settings such as rural Thailand and Laos, medical and surgical options are limited for CCA, and these options and care are complicated (Malhi and Gores, 2006, Razumilava and Gores, 2014).

Cancer of the bile ducts induced by infection with *O. viverrini* is thought to be a culmination of multi-factorial chronic inflammation and related disease processes (Plieskatt et al., 2013, Porta et al., 2011, Sripa et al., 2012). Of particular relevance to this study is the role of *O. viverrini* excretory-secretory products (ES) in establishing a carcinogenic microenvironment, including stimulating cellular proliferation of cholangiocytes lining the infected bile ducts (Smout et al., 2011, Sripa et al., 2007, Thuwajit et al., 2004). ES from *O. viverrini* stimulates the proliferation of both NIH-3T3 fibroblasts (Thuwajit et al., 2004) and the human KKU-100 cell line from a liver-fluke induced CCA (Smout et al., 2009). Using proteomic and transcriptomic approaches, *O. viverrini* ES and surface membrane proteins were characterized and shown to include a complex mixture of proteins, some of which have been associated with cancers, including proteases, protease inhibitors, orthologues of mammalian growth factors and anti-apoptotic proteins (Laha et al., 2007, Mulvenna et al., 2010). Of particular note was the identification of *Ov*-GRN-1, a protein with sequence similarity to the mammalian growth factor, granulin (Mulvenna et al., 2010, Smout et al., 2009). *Ov*-GRN-1 was expressed in diverse organs and tissues of the liver fluke, was detected on adjacent biliary epithelial cells of experimentally infected hamsters, and

antibodies raised to recombinant *Ov*-GRN-1 inhibited ES of *O. viverrini* from driving cellular proliferation (Smout et al., 2009). Hence it has become clear that *Ov*-GRN-1 is the major factor of ES, at large, that induces cell proliferation and, ultimately, likely promotes cholangiocarcinogenesis.

RNA interference (RNAi) is a natural mechanism involved with silencing the expression of genes to control homeostasis and other mechanisms (Shuey et al., 2002). This phenomenon has been co-opted in the laboratory in functional genomic approaches to analyze gene function in numerous eukaryotic species, including parasitic worms (Knox, 2012). Indeed, we successfully deployed RNAi to investigate genes in liver flukes (Rinaldi et al., 2008, Thanasuwan et al., 2014). It is notable, also, that suppression of human granulin-epithelin precursor by RNAi inhibits growth of hepatocellular carcinoma cells and shows promise as a treatment for this disease (Park et al., 2011). To further explore the role of *Ov*-GRN-1 in pathogenesis of opisthorchiasis, here we used RNAi to suppress the expression of *Ov-grn-1* in adult flukes, which lead to decreased viability of cultured flukes and compromised ability of ES to stimulate proliferation of cholangiocytes and related tumor cells.

2. Materials and methods

2.1. Opisthorchis viverrini

Metacercariae of *O. viverrini* were recovered from flesh of naturally infected fishes, as described (Laha et al., 2007, Sithithaworn et al., 1997). Syrian hamsters (*Mesocricetus auratas*) were infected with 50 metacercariae each via an intragastric tube (Sripa and Kaewkes, 2002). Hamsters were reared at the animal facility, Faculty of Medicine, Khon Kaen University. Study design protocols and standard operating procedures were approved by the Animal Ethics Committee of Khon Kaen University according to the Ethics of Animal Experimentation of the National Research Council of Thailand, approval number AEKKU43/2555. Hamsters were euthanized at six weeks after infection, at which point adult worms were recovered from the in gall bladders and bile ducts.

2.2. O. viverrini excretory-secretory products (ES) and lysate of adult worms

A soluble lysate of adult worms, which we termed somatic adult worm extract (SAE) was prepared from frozen and homogenized adult worms (Smout et al., 2009). ES was prepared from viable adult worms washed in antibiotics and incubated in modified RPMI-1640 (Life Technologies) at 37°C/5% CO_2 and the supernatant harvested daily for three days and stored at −80°C (Sripa and Kaewkes, 2000).

2.3. Recombinant protein production

Purification of *Ov*-GRN-1 recombinant protein was as our previously optimized protocol using BL21 *Escherichia coli* bacterial insoluble expression with refolding followed by pure monomer isolation (Smout et al., 2011).

2.4. Human cell culture

The non-malignant cholangiocyte cell line H69 is a SV40-transformed human bile duct epithelial cell line originally derived from a normal liver harvested for transplantation

(Grubman et al., 1994, Reid and Jefferson, 1984) and was obtained in 2010 from Dr. Gregory J. Gores, Mayo Clinic, Rochester, Minnesota. H69s were grown under similar conditions with growth factor supplemented specialized complete media (Matsumura et al., 2004); DMEM/F12 with high glucose, 10% fetal bovine serum (FBS), 1x antibiotic/antimycotic, 25 μg/ml adenine, 5 μg/ml insulin, 1 μg/ml epinephrine, 8.3 μg/ml holo-transferrin, 0.62 μg/ml, hydrocortisone, 13.6 ng/ml T3 and 10 ng/ml EGF (Life Technologies). H69 cells were grown in complete medium as described (Grubman et al., 1994). The KKU-M214 cell line, derived from a moderately differentiated type human CCA was cultured in T75cm^2 vented monolayer flasks (Corning) in complete media [RPMI 1640 (Sigma) with 10% FBS and 1x antibiotic/antimycotic as previously described (Sripa et al., 2005).

Cell lines were incubated in 5% CO_2 at 37°C, and Trypan blue staining was used to determine cell viability. Cells were gown to ~80% confluence for further experiments. All growth assays for cells were performed in low nutrient media-5% complete media. For H69s this is 0.5% with FCS with 1/20th of the previously listed growth factors for complete media. For the KKU-214 cell lines, this was RPMI 1640 medium with 0.5% with FCS.

2.5. Growth assays with xCELLigence

Cells were seeded at 3,000 cells/well in 200 μl of complete media (above) in E-plates (ACEA Biosciences, San Diego, California) and grown overnight while monitored with an xCELLigence SP system (ACEA Biosciences) which monitors cellular events in real time by measuring electrical impedance across inter-digitated gold micro-electrodes integrated on the bottom of tissue culture plates (Ke et al., 2011). Cells were washed three times with PBS and replaced with 180 μl of relevant low nutrient media and incubated for a minimum of six hours before further treatments. Treatments were prepared at 10× concentration in low nutrient media and added to a final in-well concentration of 1× (i.e., 20 μl into each well). The xCELLigence system recorded cell index (CI) readings hourly for six days post treatment. GraphPad Prism 6.02 was used for cubic or quadratic non-linear best fit regression (intercept constrained to 1) with least squares fit with comparisons between treatments and TRX controls performed with extra sum of squares *F* test with *P* values of ≤ 0.05 and $P \leq 0.001$ deemed significant and highly significant, respectively.

2.6. Ov-grn-1 *gene and double-strand RNAs (dsRNAs) synthesis*

The fragment of *Ov-grn-1*of 285 bp (spanning nt no. 49-333, GenBank accession FJ436341) was amplified by PCR from a cDNA library assembled from adult flukes (Laha et al., 2007). The target locus was amplified using specific primers flanked with T7 RNA polymerase promoter sequence (indicated in bold and italicized font) at the 5' ends; *Ov*-dsRNA-T7-Gra-F, 5'-***TAATACGACTCACTATAGGG***GTGTTGACGGTGATTTCAC-3' as forward primer and *Ov* dsRNA-T7-Gra-R, 5'-***TAATACGACTCACTATAGGG***CTTTCGAGCGTTGAGCATAA-3') as reverse primer. PCR reactions were carried out as follows: 100 ng of *O. viverrini* cDNA library as template, 0.2 mM dNTP, 1.5 mM $MgCl_2$ performed with 1 unit *Taq* polymerase (Invitrogen, Germany) with 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec and a final extension at 72°C for 10 min. PCR products were

identified by agarose gel electrophoresis and purified by gel extraction kit (Fermentas, EU). PCR products were ligated into the pGEM-T Easy vector (Promega, USA), after which *Escherichia coli* JM109 competent cells (Promega) were transformed with the ligation products. Recombinant clones were screened on ampicillin with blue/white selection. White colonies were grown in LB, and positive inserts identified by PCR using primers corresponding to the multiple cloning site promoter sequences, T7 and SP6. The extracted plasmids were subjected to sequencing using the BigDye terminator method (1st BASE, Singapore).

To synthesize *Ov-grn-1*double stranded RNA (dsRNA), amplicons were generated by PCR from the pGEM-T plasmid clone of *Ov-grn-1* (above). The PCR conditions were 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec and a final extension at 72°C for 10 min. A 285 bp fragment of *Ov-grn*-1 (as above) was generated by *in vitro* transcription using specific primers tailed with the T7 RNA polymerase promoter sequence *Ov*-dsRNA-T7-Gra-F and *Ov*-dsRNA-T7-Gra-R (described above). An irrelevant negative control firefly *luciferase* (*Luc*) dsRNA derived from pGL3 was also produced (Promega, USA). *Luc* dsRNA was amplified using primers ds-LUC_T7-F5'-***TAATACGACTCACTATAGGG***TGCGCCCGCGAACGACATTTA and ds-LUC_T7-R5'-***TAATACGACTCACTATAGGG***GCAACCGCTTCCCCGACTTCCTTA (Rinaldi et al., 2008). The PCR products were *in vitro* single transcribed to produce dsRNAs and purified using the Megascript RNAi kit (Ambion, USA) accorded to the manufacturer's instructions. Integrities of the dsRNAs were assessed on a 1.5% agarose gel and concentrations determined by spectrophotometer (NanoVue, GE Healthcare, USA).

2.7. *Delivery of Ov-grn-1 dsRNA into* O. viverrini *adult flukes*

Adult stage *O. viverrini* flukes from hamsters were washed several times with sterile normal saline and then washed five times in DMEM (Gibco, USA) containing 2× antibiotics. Flukes were incubated for 1 hour at 37°C in 5% CO_2 atmosphere with DMEM containing 2× antibiotics. The flukes were resuspended in 100 μl of DMEM plus 1x antibiotic and 50 μg of *Ov-grn-1* dsRNA in 4 mm gap cuvettes (Bio-Rad, Hercules, CA, USA) and subjected to a single pulse of square wave electroporation at 125 V for 20 milliseconds (Electroporator Gene Pulser Xcell, Bio-Rad). The parasites were soaked with 50 μg of *Ov-grn-1* dsRNA in 10 ml of 1× DMEM for two days and maintained at 37°C and 5% CO_2 in air before downstream processing.

2.8. RNA extraction and quantitative real time-PCR

To determine RNA expression levels, *Ov-grn-1* dsRNA treated worms (n = 30) were cultured in RPMI-1640 supplemented with 1x antibiotic and antimycotic, and single worms in triplicate were harvested at days 1, 2, 3, 5 and 7 to evaluate the endogenous expression of *Ov grn-1* mRNA. Flukes that were not exposed to dsRNA but were electroporated in media served as mock controls, and worms exposed to *Luc* dsRNA were used as negative, irrelevant controls. Total RNA of flukes was extracted with TRIZOL (Invitrogen, USA) following the manufacturer's instructions, and contaminating genomic DNA was removed by treatment with DNase I (Thermo-scientific, EU). Concentrations of RNA were determined with a spectrophotometer. cDNAs were synthesized from 1 μg of adult RNA

using a cDNA synthesis kit (Fermentas). *Ov-grn-1* cDNA was amplified using specific primers as follows: forward primer, *Ov*-GRN-EXF: 5'-GGGATCGGTTAGTCTAATCTCC-3', and reverse primer, *Ov*-GRN EXR: 5'-GATCATGGGGGTTCACTGTC) (spanning *Ov-grn-1* coding DNA position 6-365) amplifying a 359 bp product which is larger than the dsRNA used for electroporation and therefore cannot be amplified from exogenous dsRNA. The endogenous actin gene (GenBank AY005475) was used as a housekeeping control (Piratae et al., 2012). Real time PCR was carried out using an ABI7500 thermal cycler and the SYBR Green assay. PCR reactions consisted of 12.5 μl of SYBR Green Master Mix (TAKARA Perfect Real-time Kit),0.5 μl (10 mM) each of forward and reverse primers, 0.5 μl of reference Dye (ROX), 1 μl (equivalent to 50 ng of total RNA) of first-stand cDNA and water to a final volume of 25 μl. PCR cycling conditions consisted of initiation with pre-heat for one cycle at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for30 sec, annealing at 55°C for 30 sec, extension at72°C for 45 sec, and a final extension at 72°C for 10 min. The endogenous *Ov-grn-1* mRNA levels were normalized with actin mRNA. Data is presented as the unit value of $2^{-\Delta\Delta Ct}$ where ΔΔCt = ΔCt (treated worms)-ΔCt (non-treated worms); ΔCt of each group = $Ct_{grn\ 1,2,3} - Ct_{actin(average)}$. In addition, RT PCR products amplified from total RNA of adult flukes were run on 1.5% agarose gels in order to visually assess the integrity of the bands.

2.9. Viability of parasites

To determine the viability of parasites during *in vitro* culture, twenty worms treated with *Ov-grn-1* dsRNA were cultured in DMEM containing 1x antibiotic; media were changed every two days. Viability of flukes was determined on day 5 by observing movement of oral or ventral suckers using light microscopy in the presence of the vital dye, Trypan blue at 0.1% in PBS for 10 min at 37°C, 5% CO2. Flukes were scored as dead if the worm stained blue, or as alive if not stained or stained weakly with Trypan blue and the oral or ventral suckers was still moving (alive), or dead, if no movement was apparent during observation for 10 min duration (Gold, 1997, Rinaldi et al., 2012). Worm survival among treatment groups was compared using independent *t*-tests.

2.10. Cell proliferation assay using a non-contact co-culture technique

To evaluate the effect on cell proliferation of suppressing *Ov-grn-1*, viable *O. viverrini* adult flukes that had been electroporated once and soaked with *Ov-grn-1* dsRNAs for 24 hours and cultured in culture media for another day. Flukes were then co-cultured with either H-69 or KKU-M214 cells using 24-well Transwell plates with a 4 μm pore size membrane separating the upper and lower chambers (Corning, USA). Briefly, 10,000 of either H-69 or KKU-M214 cells were seeded into the lower chamber of the Transwell plate and cultured for 24 h with complete medium. Complete medium was removed and replaced with incomplete medium, which consisted of complete medium diluted to 3:20 in DMEM/F12 for H-69, and Ham's F-12 supplemented with 1% FBS for KKU-M214 for overnight and culture medium was renewed with incomplete medium prior to the next experiment. Five active and viable *O. viverrini* adult flukes that had been exposed to *Ov-grn-1* dsRNAs were transferred to the upper chamber of each well. In addition, five adult flukes that had been electroporated/soaked with no dsRNAs were used as a mock control. Wells without flukes served as a negative control. Four replicates of treatments were conducted. The fluke ES but

not the fluke itself could reach the lower chamber, which was separated from the upper chamber by a membrane a pose size of 4 μm diameter. The number of cells in each well was determined at days 1, 2 and 3 using the WST-1 cell proliferation reagent (Roche, Germany), where 10 μl of WST-1 was added to cells at 37°C for up to 30 min for KKU M214 and 2 h for H-69 cells. Absorbance at 450 nm was determined and cell number calculated from a standard curve (following the manufacturer's instructions) before converting into relative growth compared to the negative control. Independent *t*-tests were used to compare treatment with *Ov-grn-1* dsRNA versus the mock control.

2.11. Statistical analysis

Mean and standard deviation were calculated using SPSS for windows version 21 or GraphPad Prism software; ANOVA and Student's *t*-test were used to compare groups. $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Fluke proteins stimulate hyper-proliferation of human cholangiocytes

We compared the effect of *Ov*-GRN-1 in stimulating human cholangiocytes (H69s) using recombinant *Ov*-GRN-1, in comparison with a soluble lysate of worms and with ES of *O. viverrini*, using real time cell growth analysis using the xCELLigence system (Figure 1). All four treatments induced cellular proliferation, when compared with controls (PBS or TRX protein) ($F_{(DFn, DFd)}$ = 487.5-5253 $_{(2,1478)}$). Cellular growth was monitored for up to 140 hours after treatment, but only up until 90 hours was used for comparisons since the low nutrient media causes control cells numbers to decline. The two controls, PBS vehicle control and the expression matched bacterial TRX, showed similar growth rate curves. It is noted that three of the treatments showed similar profiles, 12 nM *Ov*-GRN-1, 14 μg/ml ES of *O. viverrini* and lysate of worms (SAE), climbed above controls after about one day and reached 27-38% over controls at 90 h. These comparable rates of induction of proliferation allowed approximation that the proportion of *Ov*-GRN-1 to represent ~0.8% of the total protein in ES. This assumes that recombinant *Ov*-GRN-1 was fully active and that are no other factors from the flukes influenced cell growth-as shown previously by Smout *et al*. (Smout et al., 2009). The lower dose treatment of 3 nM *Ov*-GRN-1 also significantly induced growth, compared to control wells, but at a far lower rate of 13% at 90 h ($F_{(DFn, DFd)}$ = 487.5 $_{(2,1478)}$).

3.2. *Suppression of* Ov-grn-1 *expression in* Opisthorchis viverrini *by RNAi*

In order to investigate the role of *Ov-grn-1* by suppressing its expression via RNAi, we subjected adult flukes to square wave electroporation of *Ov-grn-1* or *Luc* dsRNA (or media alone) and then, in addition, soaked the worms for two days in dsRNA. Total RNA was extracted from the flukes and used as template for quantitative RT-PCR. Potent knockdown of *Ov-grn-1* resulted in flukes treated with *Ov-grn-1* dsRNA compared to controls (Figure 2). Significant knockdown of *Ov-grn-1* gene expression compared to mock controls ($P \leq 0.001$) was evident as early as 48 h after electroporation (91.4% reduced expression), which progressively increased to complete knockdown by day 7 (Figure 2). Thereafter, the majority of the flukes electroporated with *Ov-grn-1* dsRNA were dead; and accordingly

transcript levels were only considered for the first seven days of culture (below). A reduction in the expression of *Ov-grn-1* expression in flukes that were electroporated in *Luc* dsRNA (Figure 2) was not seen. Moreover, differences in expression of the *actin* gene among the groups were not apparent (not shown). RT-PCR products amplified from total RNA of adult that were analyzed on 1.5% agarose gel and showed less intensity corresponding to knockdown level compared to actin which were not different (Figure 2). These findings confirmed the experimental RNAi silencing of *Ov-grn-1* at the transcript level.

3.3. *Suppression of* Ov-grn-1 *mRNA hastened death of worms*

To determine the effect of silencing the expression of *Ov-grn-1* on viability of the flukes, we determined viability rates by observing motility and death by Trypan blue exclusion. Viability decreased significantly in *Ov-grn-1* dsRNA-treated worms compared to the mock control group. More specifically, *Ov-grn-1* dsRNA-treated flukes exhibited significantly decreased (40%) viability when compared to intact, control worms by five days of culture in standard medium (not containing dsRNAs) (Figure 3).

3.4. *Silencing of* Ov-grn-1 *results in reduced ability of* O. viverrini to drive cell proliferation in Transwell co-culture

To further explore the role of *Ov*-GRN-1 in driving proliferation of human host cells, *Ov-grn-1* knocked-down worms were co-cultured with cholangiocytes (H-69) and a line of CCA cells (KKU-M214). Compared to electroporation mock control parasites, *Ov-grn-1*-suppressed parasites induced far less cell proliferation of H-69 ($P \leq 0.001$) and KKU-M214 ($P \leq 0.001$) after as brief a period as 24 h in culture (Figure 4A and 4B). After three days of cell culture, highly significant inhibition of proliferation continued ($P \leq 0.001$).

4. Discussion

The mechanisms by which infection with *O. viverrini* results in liver disease and chronic infection can ultimately cause CCA are undoubtedly complex, involving multi-factorial mechanisms (Plieskatt et al., 2013, Sripa et al., 2012). Chronic irritation caused by *O. viverrini* induces inflammation of the bile ducts, resulting in hyperplasia of exposed biliary epithelial cells. The induction of cell proliferation is an important step in the genesis of cancer because hyperplastic cells are vulnerable to a carcinogen that can easily induce DNA damage during mitosis and proliferation (Watanapa and Williamson, 1993, Williamson and Rainey, 1984). The mechanisms involved in these phenomena are multifactorial, and may include 1) mechanical, where direct contact of parasites with the bile duct epithelium results in constant abrasion and irritation, and 2) direct and varied effects of ES from the flukes (Sripa et al., 2007) stimuli, among others (Porta et al., 2011). ES from *O. viverrini* can induce proliferation of fibroblasts (Thuwajit et al., 2004) as well as in KKU-100 human cholangiocarcinoma cells (Smout et al., 2009). ES includes a complex catalogue of proteins that have been associated with cancers, particularly the potent growth factor *Ov*-GRN-1 (Mulvenna et al., 2010).

Human granulin is expressed as pro-granulin, PGRN, and is a secreted glycoprotein comprised of seven tandem repeats of a 12-cysteine module called granulin A-G or epithelin domains. It is sensitive to hydrolysis by elastase, which liberates peptides of 6 kDa corresponding to individual granulin modules that maintain diverse biological activity (Bateman and Bennett, 1998, Tolkatchev et al., 2008). In addition to the granulin units initiating signal cascades, intact PGRN has key biological activities, including regulation of early embryogenesis, repair of adult tissues and modulation of inflammation. PGRN is involved in multiple steps in the tumor progression cascade, including cellular proliferation, tissue repair, and tumorigenesis (He and Bateman, 2003). For example, treatment of CCA cells with recombinant PGRN results in increased cell proliferation *in vitro*, and suppression of PGRN expression in CCA cells decreases the expression of proliferating cellular nuclear antigen, a marker of proliferative capacity, and retards tumor growth *in vivo* (Frampton et al., 2012). Moreover, PGRN promotes migration of 5637 bladder cancer cells and stimulates wound closure and invasion (Monami et al., 2006). Nonetheless, the signaling cascade is not fully understood since either or both of PGRN or the individual granulin A-G units may be responsible for initiating proliferation.

Earlier findings revealed that *Ov*-GRN-1 stimulates proliferation of murine fibroblasts, and proliferation is inhibited by the MAPK kinase inhibitor, U0126 (Smout et al., 2009). Antibodies raised to recombinant *Ov*-GRN-1 inhibit the ability of ES to induce proliferation of murine fibroblasts and a human CCA cell line *in vitro*, indicating that *Ov*-GRN-1 is the major growth factor present in *O. viverrini* ES products (Smout et al., 2009). In the present study, we silenced the expression of the *Ov-grn-1* gene in adult flukes using RNA interference, and noted a suppression of mitogenicity in the ES products of the fluke. An intriguing finding was the increased lethality that follows silencing of *Ov-grn-1*. This implies that *Ov*-GRN-1 acts as a growth factor for both host cells and the fluke itself. Indeed, to our knowledge, this is the first report describing the silencing of a growth factor gene in a parasitic helminth. The ubiquitous expression of *Ov*-GRN-1 in organs and tissues of adult *O. viverrini* (Smout et al., 2009) supports the notion that this fluke mitogen functions as an essential, endogenous growth factor for *O. viverrini*. It is not unexpected that *Ov*-GRN-1 functions as a growth factor for both the liver fluke and its host cells since granulins evolved in all branches of the animal kingdom and are known to promote proliferation of different cell types from phylogenetically distant taxa (Hanington et al., 2008). Future studies will explore the effects of introducing *Ov-grn-1* dsRNA-treated metacercariae (the infectious stage to mammals) to hamsters to assess survival *in vivo*.

In terms of infiltration of dsRNAs throughout fluke tissues during the RNAi process, notably, electroporation with as little as 50 μg of dsRNA per 100 μl of media followed by soaking in 50 μg/10 ml for two days was sufficient to see significant knock down of *Ov-grn-1* in adult flukes, and resulted in death of some flukes by five days of culture. Andrew *et al* (1998) reported that only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process (Fire et al., 1998). Previously, electroporation of Cy3-siRNA into adult *O. viverrini* revealed the location of the reporter siRNA throughout the parenchyma of transduced

worms, with accumulation in the gut (Sripa et al., 2011). Furthermore, *Ov*-GRN-1 was detected in fluke gut tissue and ES products, as well as on the surface of biliary epithelial cells of hamsters that were experimentally infected with *O. viverrini* (Smout et al., 2009), underscoring extracellular roles for the protein at the host-parasite interface. Taken together, we suggest that dsRNAs introduced into *O. viverrini* adults enter into apertures on the body of the fluke including the tegument, genital pore and mouth from where they pass through the gut. Presumably the affected cells amplify the component in the interference process, interfering with target gene expression.

In the human liver, differentiated cholangiocytes are mitotically dormant (Priester et al., 2010). Cholangiocyte proliferation may include some combination of proliferation of pre existing ductules, progenitor cell activation, and appearance of intermediate hepatocytes (Priester et al., 2010). Proliferation of cholangiocytes induced by the fluke infection may serve to compensate for cells damaged by the fluke and maintain secretory function (Leite and Nathanson, 2005). Therefore, inhibiting the activity of *Ov*-GRN-1 may decrease excess cell proliferation and potentially inhibit the development of biliary fibrosis or malignancy (Ho et al., 2008, Smout et al., 2009). Hence, *Ov*-GRN-1 may be plausible target of a potential anti-parasitic and/or anti-pathogenic vaccine to control the opisthorchiasis and opisthorchiasis-induced cholangiocarcinoma.

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HIGHLIGHTS

>Granulin of *Opisthorchis viverrini* is susceptible to experimental RNAinterference

>Suppression of granulin reduced ability of ES products to drive cell proliferation

>Knock down of granulin hastened death of cultured liver flukes

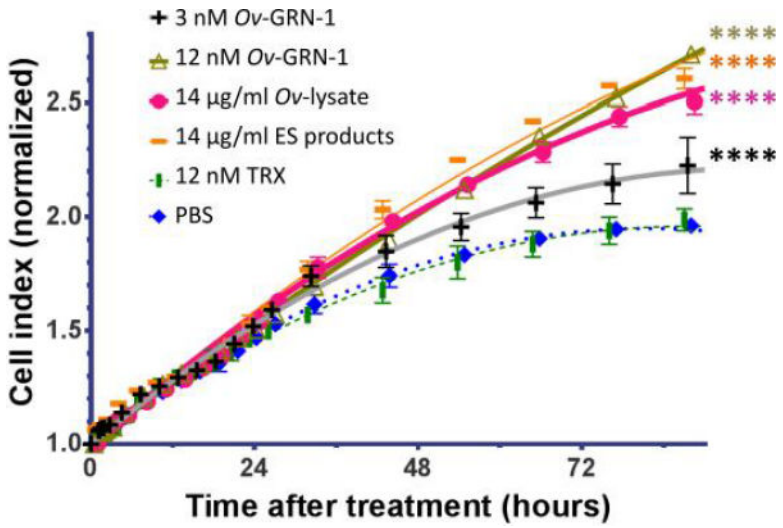

Figure 1. Proteins from *Opisthorchis viverrini* stimulate the proliferation of human cholangiocytes *in vitro*

Normalized Cell Index is the output from the xCELLigence instrument. *Ov*-GRN-1 is produced in *E. coli* expressed and refolded into an active growth factor. The lysate of *O. viverrini* represents a soluble extract of adult *Opisthorchis viverrini* worms. *Ov*ES (excreted/secreted products) is the supernatant from cultured adult *O. viverrini*. TRX is thioredoxin from *E. coli* and is an expression-matched control for *Ov*-GRN-1. Error bars reveal the standard error of triplicate biological replicates. **** denotes statistical significance at the level of $P \leq 0.0001$.

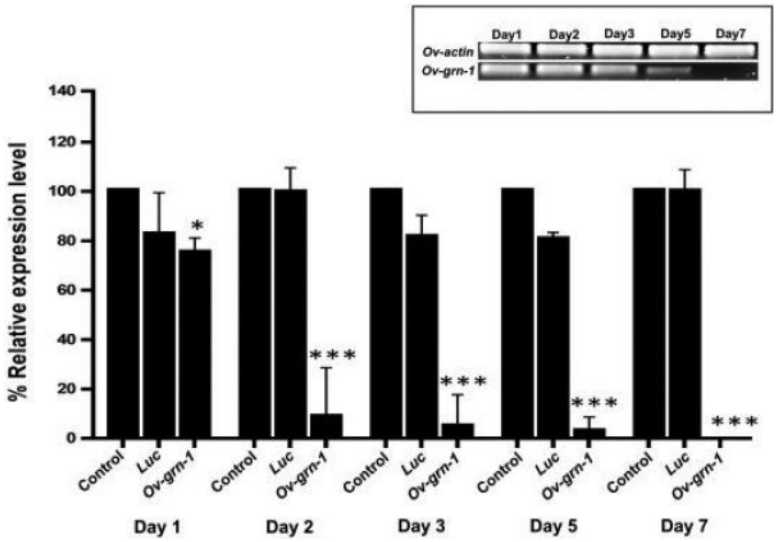

Figure 2. Gene-specific silencing of *Ov-grn-1* in *Opisthorchis viverrini* following delivery of dsRNA by square wave electroporation

Real-time qPCR analysis revealed the transcript levels of *Ov-grn-1* normalized to levels of *Ov-actin* in flukes after electroporation and soaking with *Ov-grn-1* or *luciferase* (*Luc*) dsRNAs. *Ov-grn-1* transcript levels had decreased significantly by day 1 and were not detectable by day 7. *Ov-grn-1* RT-PCR products amplified from total RNA of adult flukes after electroporation with either *Ov-grn-1*or *Luc* dsRNAs (inset). Data are represented by mean ± 1 standard deviation; *** denotes statistical significance at the level of $P \leq 0.001$.

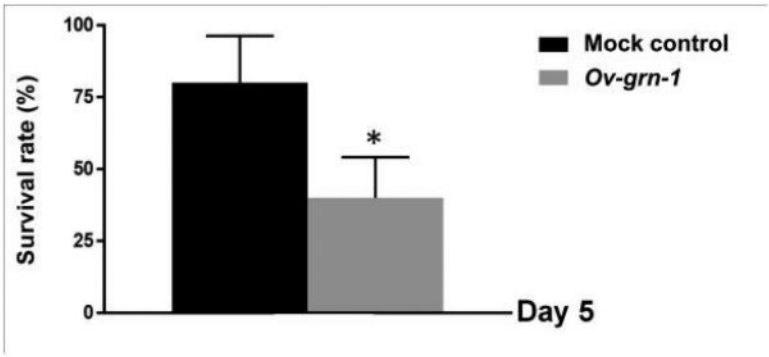

Figure 3. Survival rate of *Opisthorchis viverrini* after RNAi-based silencing of *Ov-grn-1*

Twenty adult flukes were cultured for five days. The worms were examined the viability with Trypan blue by light microscopy. Data were represented by mean ± 1 standard deviation. Significance at the level of $P \leq 0.05$ level denoted with * in a comparison with flukes not exposed to dsRNA but subjected to identical electroporation (mock).

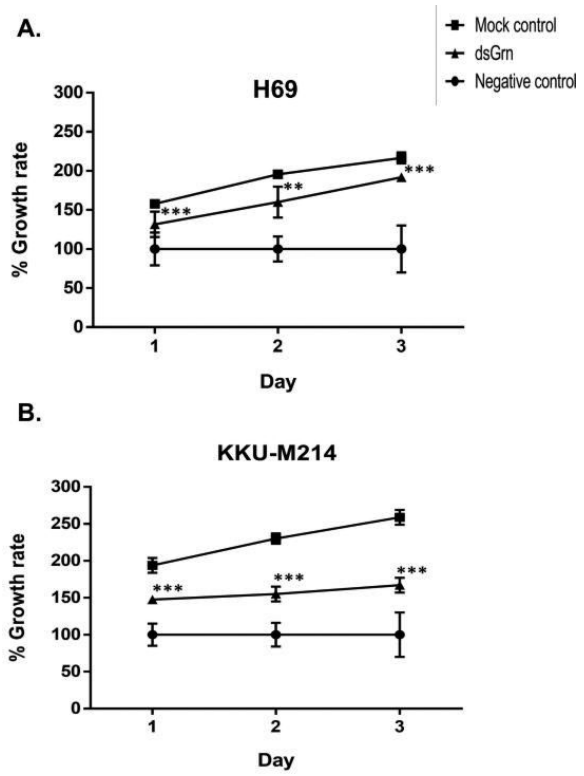

Figure 4. Proliferation of cholangiocytes repressed after exposure to *Opisthorchis viverrini* subjected to gene knockdown of *Ov-grn-1*

Growth expressed as the ratio of cell numbers per well between treatment and control groups ("no parasites" used as 100% negative control). The effect of specific repression of expression of *Ov-grn-1* on growth of lines of immortalized cholangiocytes (H69 cell line) (panel A) and cholangiocarcinoma cells (KKU-M214 cells) (Panel B) resulted in significantly decreased proliferation compared to controls during cultivation for three days. Findings presented as the means of biological quadruplicates, with the standard deviation shown as bars. Asterisks indicate significant differences between mock control and *Ov-grn-1* dsRNA treatments: ** and *** represent $P \leq 0.01$ and 0.001, respectively.