CHAPTER III
FUNCTIONAL ANALYSIS OF *OPISTHORCHIS VIVERRINI* CATHEPSIN B USING RNA INTERFERENCE

3.1 Introduction

Throughout East Asia, there is a strikingly high prevalence of cholangiocarcinoma (CCA), cancer of the bile ducts, in regions where the human liver fluke is endemic. No stronger link occurs between a human malignancy and infection with a eukaryotic parasite than that between CCA and infection with the liver fluke, *Opisthorchis viverrini* (Digenea) (reviewed in (Sripa et al., 2007). Indeed, the International Agency for Research on Cancer (IARC) recognizes *O. viverrini* as a 'group 1 carcinogen' (Bouvard et al., 2009). CCA is prevalent in Northeast Thailand, a region where uncooked cyprinoid fish is a dietary staple. Due to poor sanitation practices and inadequate sewerage infrastructure, *O. viverrini*-infected people pass the fluke eggs in feces into natural bodies of fresh water. Aquatic snails, the first intermediate hosts of *O. viverrini*, ingest the eggs from which the miracidia undergo asexual reproduction before a population of the free swimming larval stage, called a cercaria, is shed from the infected snails. The cercaria locates a cyprinoid fish and encysts in the fins, skin and musculature as a metacercaria. Metacercariae of *O. viverrini* are infective to humans and other fish-eating mammals. Infection occurs when people ingest raw or undercooked fish. In Thailand and Laos, ~10 million people are infected with *O. viverrini.* Despite widespread chemotherapy with praziquantel, the prevalence of *O. viverrini* in some endemic areas approaches 70% (see (Sripa and Pairojkul, 2008)). Moreover, in Thailand, liver cancer is the most prevalent of the malignant/fatal neoplasias, and the prevalence of CCA in regions in which the parasite is endemic is unprecedented (Srivatanakul, 2001).

The mechanisms by which *O. viverrini* infection causes cancer are likely to be multi-factorial (Sripa et al., 2007). The parasite induces inflammation of the bile ducts, resulting in oxidative DNA damage of the epithelium. In addition, the flukes secrete proteins that promote biliary epithelial cells to hyper-proliferate but not

undergo apoptosis, providing an additional potential mechanism by which epithelial cells become malignant (Pinlaor et al., 2004). Characterization of these secreted antigens may give important information about the key molecules responsible for *O. viverrini*-associated CCA pathogenesis (Pinlaor et al., 2009; Smout et al., 2009; Sripa et al., 2010b). Secreted proteases have been found in abundance in *O. viverrini* secretions where they play key roles in food digestion and may be involved in bile duct inflammation. *O. viverrini* cathepsin F (*Ov*-CF-1) and cathepsin B (*Ov*-CB-1) were shown as the key enzymes in food catabolism (Pinlaor et al., 2009; Sripa et al., 2010b). In addition, the *Ov*-CB-1 is important in regulating the activity of *Ov*-CF-1 and that both enzymes work in concert to degrade host tissue macromolecules. Damage caused by the concerted action of these two cathepsins on the epithelium of the biliary tree may contribute the development of liver fluke associated CCA. Its role suggest it may be developed as an intervention target e.g. vaccine or drug target, in like fashion to cathepsin B of the human blood fluke *Schistosoma mansoni* (Abdulla et al., 2007). In addition, there have major developments in transcriptomics of *O. viverrini* and *C. sinensis* (Laha et al., 2007; Young et al., 2010) but there are no reports on the availability of gene manipulation approaches for *O. viverrini* and other related species.

Here we have targeted cathepsin B of *O. viverrini* as a model gene for development of RNAi for *O. viverrini* and other fish-borne flukes. We show that cathepsin B is susceptible to RNAi knockdown, as evidenced by both reductions in transcript levels and indeed in enzyme activity ascribable to cathepsin B.

3.2 Materials and Methods

3.2.1 Adult of *O. viverrini* and cultivation

Metacercariae (MC) of *O. viverrini* were obtained from naturally infected cypriniid fish by pepsin digestion, as detailed (Tesana et al., 1985). Syrian golden hamsters, *Mesocricetus auratus*, were fed fifty MC by orogastric tube. Infected hamsters were euthanized six weeks after infection and adult *O. viverrini* flukes recovered from the gall bladder and bile ducts. Worms were washed for five times with PBS supplemented with 2X antibiotics (streptomycin/penicillin, 200 μg/ml), and

then cultured in RPMI medium supplemented with 1X antibiotics (streptomycin/ penicillin, 100 μg/ml) at 37°C under 5% CO_2 in air.

3.2.2 Synthesis of *O. viverrini* cathepsin B dsRNA

Double stranded-RNA of the *Ov*-CB-1 gene (residues 257-753 of the transcript, 473 bp) was synthesized from DNA templates encoding *O. viverrini* cathepsin B1 (GenBank accession GQ303560). The plasmid clone of *Ov*-CB-1 in TOPO vector (Invitrogen, USA) was used as DNA template for dsRNA of *Ov*-CB-1 using gene-target primers containing T7 promoter sequence (Fwd : 5'-*TAA TAC GAC TCA CTA TAG GG*C ACG GGA ACA ATG GCC TCA CTG TC-3' ; Rev : 5'-*TAA TAC GAC TCA CTA TAG GG*C GCT GCT TCG ACT GGT CCG TTG AT-3'). Freshly synthesized cDNA from adult *O. viverrini* (Laha et al., 2007) was employed as the template for dsRNA specific for the granulin (*Ov-grn-1*) gene (Smout et al., 2009) using gene-targeting primers containing T7 promoter sequences, 5' *TAA TAC GAC TCA CTA TAG GG* TTACGGATGCTGTCCTATGG (Fwd) and *TAA TAC GAC TCA CTA TAG GG* CTTTCGAGCGTTGAGCATAA (Rev). The DNA containing the T7 RNA polymerase promoter sequence at each end (as indicated in italics) was used as templates for synthesized dsRNA. dsRNAs were synthesized and purified with the Megascript RNAi kit (Ambion, Austin, TX, USA), according to manufacturer's instructions. The concentration and purity of dsRNAs were determined with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.2.3 Electroporation of dsRNA and siRNA

O. viverrini adult flukes were washed with four times with PBS containing penicillin and streptomycin (100 U, 100 μg /ml) and then incubated in RPMI containing antibiotics for 30 min at 37°C under 5% CO_2 in air, prior to electroporation. Silencer® Cy™3 labeled siRNA (Ambion) was used to test efficacy of square wave electroporation to deliver exogenous nucleic acid probes into the tissues and organs of *O. viverrini*. In brief, five μg of Silencer Cy3-labeled siRNA in 100 μl of RPMI supplemented with 1% glucose, 2 μM of E64 and streptomycin /penicillin (100 μg, 100 U/ml) was delivered to adult worms in 4 mm gap electroporation cuvettes (BTX, San Diego, CA, USA). Electroporation was performed at 125 V, 20 ms square wave with one pulse, using an Electroporator Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA). Pre-warmed culture medium was added after

electroporation after which transformed flukes were cultivated at 37°C under 5% CO_2 in air. Transformed flukes were examined 0, 24 and 48 h later by bright field and fluorescence microscopy.

To deliver dsRNA targeting cathepsin B by electroporation into adult *O. viverrini*, 30 flukes in 100 µl of RPMI 1640 medium with supplements (above) were dispensed into a 4 mm gap electroporation cuvette (BTX) along with 100 µg dsRNA targeting *Ov*-CB-1 added. Electroporation was performed as above, after which the transduced flukes were transferred into RPMI 1640 culture media at 37°C under 5% CO_2 in air. Culture media were replaced at intervals of three days. Worms were harvested one, two or three days later for investigation of *Ov*-CB-1 , *Ov*-CB-2, *Ov-grn-1* (granulin) and actin RNA expression or harvested at one, two, three, six or nine days later in order to examine cathepsin B protease activity.

3.2.4 RNA extraction and reverse-transcription PCR

Total RNA was isolated from adult *O. viverrini* flukes using Trizol® (Invitrogen). Total RNA was treated with DNase I (Fermentas, Vilnius, Lithuania) to remove any contaminating DNA at 37 °C for 30 min and inactivated at 65°C for 10 min. RNA concentration was determined using a spectrophotometer (ND-1000, NanoDrop Technologies). First-strand cDNA was synthesized from 1 µg of DNase-treated RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Expression of *O. viverrini* genes, *Ov*-CB-1, *Ov*-CB-2, granulin *Ov-grn-1* and *actin* was investigated using both conventional RT-PCR and RT-quantitative PCR (RT-qPCR) using the following primers; for *Ov*-CB-1, Fwd, 5'- CAGCTGCGGG TGAAGTTACAGGAT and Rev, 5'- GGTCTTGGGTATGTTTTCATCGC, which span positions nt 38-249 of the *Ov*-CB-1 transcript (above). (These primers were designed to anneal outside of the target sequences of the dsRNA of *Ov*-CB-1 interference to avoid cross contamination). The specific primers for detecting *Ov*-CB-2 (GenBank GQ303559) transcripts, residues 51-264, 213 bp were CCAAGACG CCCAGTGTGGAGA (Fwd) and CTTTGGGAGACGCGTATCATC (Rev); and for *Ov-grn-1*, (residues 39-142; 104 bp) were TGCAACAACTTTCTCGATGG (Fwd) and GTAAGCCGCGACAACAAGTC (Rev). Amplifications were undertaken with *Taq* polymerase (Invitrogen) in 40 cycles of denaturation at 94°C for 30 sec, annealing

at 55°C for 30 sec and extension at 72°C for 1 min, following by a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis through ethidium-stained agarose (1%) gels. RT-qPCR was performed using the SYBR Green method and a Mx3005P Real-time-PCR System (Stratagene, Agilent Technologies Inc, Santa Clara, CA, USA). Real time reactions were carried out with 2XBrilliant SYBR® Green QPCR Master Mix (Stratagene) and, performed in triplicate, as follows: initial pre-heat cycle at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. Expression levels of the *Ov*-CB-1 mRNA (and *Ov*-CB-2; and *Ov-grn-1*) and actin mRNA (OvAE1657, GenBank EL620339.1) (Laha et al., 2007), with actin included as an internal control, were determined. The mRNA level of *Ov*-CB-1 (or *Ov*-CB-2, or *Ov-grn-1*) was normalized with actin mRNA and presented as the unit value of $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct$ (treated worms) – ΔCt (non-treated worms) (Schmittgen and Livak, 2008).

3.2.5 Determination of cathepsin B activity

Soluble extracts of *O. viverrini* flukes, prepared as previously described (Sripa and Kaewkes, 2000; Wongratanacheewin et al., 1988) , were assayed for cathepsin B protease activity using a peptide substrate diagnostic for cathepsin B, Z-Arg-Arg-amino-methylcourmarin (AMC) (Sigma-Aldrich, St. Louis, MO, USA). Release of AMC from hydrolyzed substrate was measured at 348 nm excitation and 440 nm emission at 37°C continuously for 300 min using a Spectra Max Gemini XPS fluorescence plate reader (Molecular Devices Inc., Sunnyvale, CA, USA). Optimal conditions for cleavage were determined by incubating 50 µg of the soluble fluke extracts or 5 µg of recombinant *Ov*-CB-1 enzyme, prepared as described (Sripa et al., 2010b), with substrate Z-Arg-Arg-AMC to a final concentration of 2 µM in 100 mM sodium acetate (pH 5.0), 1 mM dithiothreitol, 1 mM EDTA (not shown). Because limited information was available on the specific activity of *Ov*-CB-1 in lysates of *O. viverrini*, the performance of the diagnostic substrate Z-Arg-Arg-AMC in monitoring this protease in lysates that would include other enzymes that might also cleave the substrate, and/or the performance of RNAi targeting *Ov*-CB-1, we monitored hydrolysis of Z-Arg-Arg-AMC over five hours, aiming to characterize potentially early and later proteolytic activity.

3.3 Results

3.3.1 Transduction of *Opisthorchis viverrini* with siRNA by square wave electroporation

To investigate whether *O. viverrini* flukes might be amenable to genetic manipulation, adult parasites were subjected to square wave electroporation in the presence of fluorescent labeled short interfering RNA. At 0 (Figure 3.1A, B), 24 (Figure 3.1C, D) and 48 h (Figure 3.1E, F and G, H) after electroporation with Cy3-Silencer siRNA, the transduced flukes were examined by fluorescence microscopy. Cy3-siRNA fluorescence was evident at 24 h (Figure 3.1C, D) and was more intense at 48 h (Figure 3.1E, F and 3.1G, H). Cy3-fluorescence was evident throughout much of the adult flukes, in particular in the vicinity of the oral sucker, ventral sucker, uterus and vitelline glands. The results confirmed that utility of square wave electroporation to introduce exogenous nucleic acid probes into this liver fluke and the utility of Cy3-RNA to indicate location of the reporter siRNA in the transduced worms.

3.3.2 Double stranded RNA suppresses transcription of *O. viverrini* cathepsin B

Following the demonstration of introduction of Cy3-siRNA into adult flukes using the square wave electroporation protocols pioneered by Correnti and Pearce, 2004 (Correnti and Pearce, 2004), we employed electroporation to transform adult *O. viverrini* parasites with dsRNAs specific for the gene encoding cathepsin B, *Ov*-CB-1 and, in a second group of worms, the gene encoding granulin, *Ov-grn-1*. Thereafter, in both groups, expression levels of *Ov*-CB-1, *Ov*-CB-2, *Ov-grn-1* and *actin* were investigated by qRT-PCR of total RNAs collected at 1, 2 and 3 days after exposure by electroporation to the dsRNAs. Strong knockdown of *Ov*-CB-1 was seen, >90% at each of days 1, 2 and introduction of the dsRNA. We also saw strong knockdown of *Ov*-CB2 on 1 and 2. By day 3, only ~50% knockdown of *Ov*-CB-2 was apparent. No silencing of granulin or actin was evident.

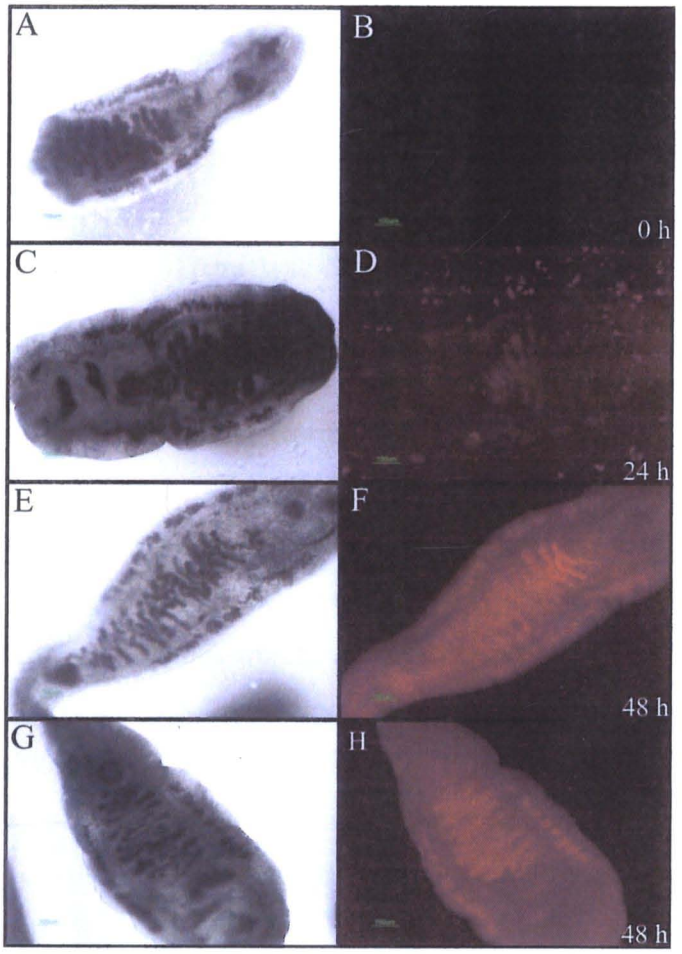

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Figure 3.1 *Opisthorchis viverrini* flukes transduced by square wave electroporation with fluorescent, control Cy3-Silencer siRNA. Adult *O. viverrini* transfected with Cy3-siRNA using square wave electroporation, employing a single pulse for 20 ms of 125 V in the presence of 50 μg/ml of Cy3-siRNA. The worms were observed under bright field (panel A, C, E and G) and fluorescence (panel B, D, F and H) at 0, 24 and 48 h after electroporation; 0 h, A, B; 24 h, C, D; 48 h, E, F, G, H. Panels E and G present two different worms at 48 h after electroporation; the two different panels are included to provide a more complete representation of the signals evident in the worms at this time point. (x4 magnification, Nikon Eclipse TS100 with Epi-Fluorescence Attachment (Mercury Lamp Illuminator model name, C-SHG) (Nikon Instruments Incorporation, Japan).

After treatment of the worms with dsRNA targeting *Ov*-CB-1, we monitored the levels of transcripts for *Ov*-CB-1 (the cognate gene target) and also three other genes, *Ov*-CB-2, *actin* and *Ov-grn-1*. We found significant silencing of *Ov*-CB-2 with dsRNA specific for *Ov*-CB-1, but no effect on two unrelated inducible genes, actin and granulin. There was a near total (>90%) reduction of *Ov*-CB-1 transcripts at one day after exposure to dsRNA. Likewise, near total knock-down in expression of *Ov*-CB-1 was maintained at two and three days after transduction (Figure 3.2). Also, we treated other worms with dsRNA targeting granulin, and then monitored expression levels of granulin (*Ov-grn-1*), cathepsin B1 (*Ov*-CB-1), cathepsin B2 (*Ov*-CB-2), and *actin*. When granulin was targeted, we saw a modest knockdown of granulin on day 1. However, by day 2, granulin expression levels had returned to levels before dsRNA treatment. By contrast, no effect was seen on *Ov*-CB-1, *Ov*-CB-2 or *actin* levels. In summary, the outcome of this control knockdown experiment suggested that off-target effects of RNAi may not be a widespread problem in functional genomics analysis in *O. viverrini*. Furthermore, we repeated the experiment, and cultured the worms for nine days after exposure to dsRNA. We observed that the near total silencing of *Ov*-CB-1 remained in place at nine days (data not shown).

In addition to demonstrating specific knockdown of *Ov*-CB-1, these findings indicate, notably, the presence of active RNAi machinery in *O. viverrini*.

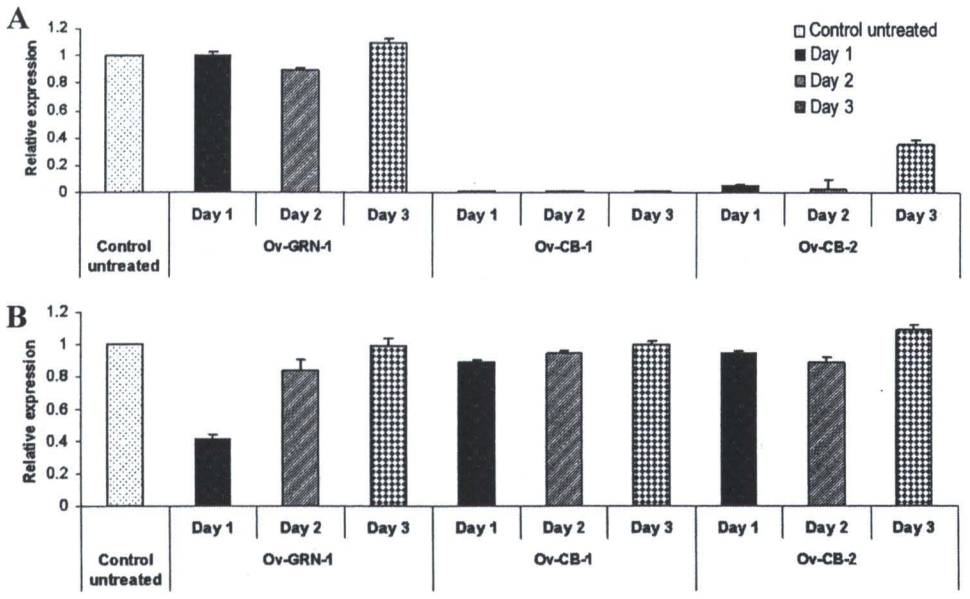

Figure 3.2 Specific silencing of expression of the cathepsin B1 gene, *Ov*-CB-1 of *Opisthorchis viverrini* by dsRNA. Panel A: Real-time RT-PCR targeting *Ov*-CB-1cathepsin B revealed >90% knockdown in specific mRNA levels when monitored at one, two and three days after introduction of dsRNAs into cultured flukes. Knockdown of *Ov*-CB-2 was also seen at days one and two. By day three, only incomplete silencing of *Ov*-CB-2 remained apparent, in contrast to the >90% knockdown of *Ov*-CB-1, indicating the silencing of *Ov*-CB-2 had commenced to wane. Panel B: In like fashion, dsRNA induced silencing of the granulin gene, *Ov-grn-1* was examined. This experiment was included here as a control to examine whether other genes in addition to *Ov*-CB-1 could be silenced. It also investigated whether silencing in *O. viverrini* was specific since the levels of *Ov*-CB-1 and *Ov*-CB-2 were examined here as well. These latter two genes appear unrelated in sequence and function of *Ov-grn-1*. For both panels A and B, relative mRNA expression levels of the *Ov*-CB-1, *Ov*-CB-2 and *Ov-grn-1* mRNA were calculated by comparing the dsRNA treated group to the non-treated group. The mRNA levels were normalized by comparison to actin mRNA and presented as the unit value of $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct$ (treated worms) – ΔCt (non-treated worms).

3.3.3 Robust knockdown of cathepsin B enzyme activity

Following qRT-PCR analysis that confirmed silencing of the *Ov*-CB-1 gene (above), other transformed flukes were assayed for cathepsin B protease activity at one, two and three days after treatment with dsRNA. In like fashion to the findings at 1, 2 and 3 days (Figure 3.2), ethidium bromide-stained gel analysis of RT-PCR products in control flukes and in flukes at one, two and three days after transfection revealed a steady decline of *Ov*-CB-1 transcripts following the electroporation of dsRNA specific for *Ov*-CB-1 (Figure 3.3A). Hydrolysis of the cathepsin B diagnostic peptide Z-Arg-Arg-AMC was undertaken using extracts of the transformed flukes, collected at one, two and three days after exposure to the dsRNA. Liberation of AMC was monitored at five min intervals for 300 min, which revealed significantly less cathepsin B activity in the transduced worms than in the control, electroporated flukes (Figure 3.3B). The difference between the control and dsRNA-treated flukes was evident in each of the three treatment groups, i.e. worms collected at one, two or three days after electroporation. More particularly, cathepsin B activity was substantially reduced by day one (60% reduction compared to control, non-dsRNA exposed flukes); at days two and three, cathepsin B activity was reduced by 45% and 40%, respectively, compared to controls (Figure 3.3). Recombinant *Ov*-CB-1 (5 μg) showed similar activity against Z-Arg-Arg-AMC to the soluble extract (5 μg) (not shown). The assay monitors hydrolysis of a fluorgenic, diagnostic peptide substrate. The differences in signals among the groups increased over time. We interpret this to reflect relatively long half-life of the cleaved products and accumulating amounts of the hydrolysis liberation products. The reduction of cathepsin B protease activity in the *Ov*-CB-1 knockdown worm group correlated with the reduction of cathepsin B mRNA levels shown by RT-qPCR and RT-PCR (Figures 3.2 and 3.3A).

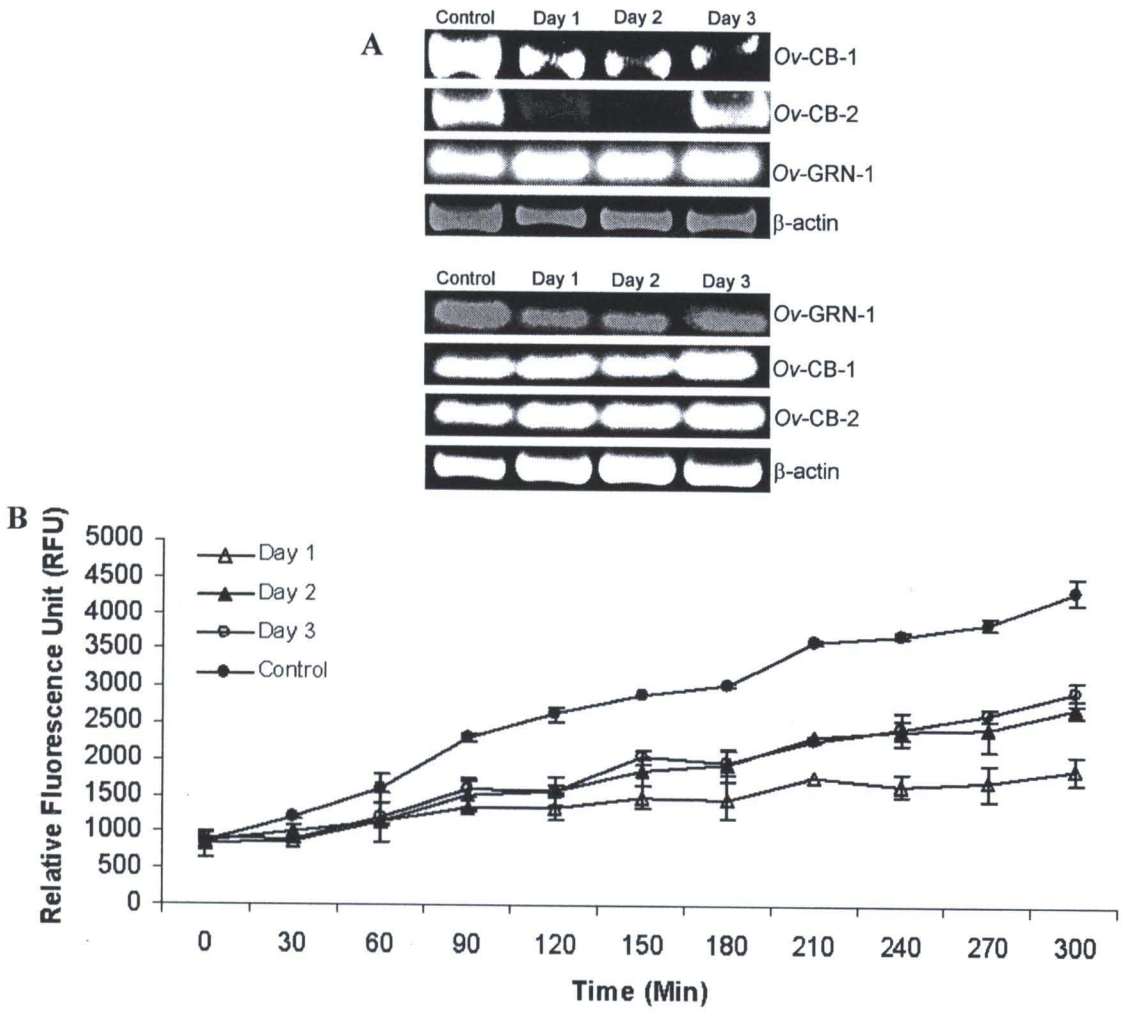

Figure 3.3 Suppression of cathepsin B activity in *Opisthorchis viverrini* flukes by treatment with dsRNA specific for *Ov*-CB-1. Reduction in expression of *Ov*-CB-1 in adult *O. viverrini* flukes transduced by square wave electroporation with 100 μg of dsRNA targeting the *Ov*-CB-1 gene at one, two and three days after treatment (panel A). Corresponding knockdown in cathepsin B protease activity observed at one, two and three days after exposure to dsRNA. Hydrolysis of Z-Arg-Arg-AMC was monitored continuously from 0 to 300 min after addition of soluble extracts of the flukes to the substrate (panel B).

3.4 Discussion

RNA interference (RNAi) is a key tool to investigate gene function and has been widely used in many eukaryotic pathogens including schistosomes (Geldhof

et al., 2007; Stefanic et al., 2010). The human liver fluke *O. viverrini* is endemic in Thailand, Laos and Cambodia where long standing infection is associated with cancer of the bile ducts, cholangiocarcinoma. Although *O. viverrini* infection remains a neglected tropical disease, recent advances on its transcriptome and proteome have provided an enormous new catalogue of sequences from which it will now be possible to develop new interventions (Laha et al., 2007; Mulvenna et al., 2010; Young et al., 2010). However, functional genomics have not been reported for *O. viverrini* or the related *C. sinensis*. We now have described gene manipulation approaches for *O. viverrini*, including the deployment of square wave electroporation to introduce small RNAs into adult *O. viverrini* obtained from experimental infected hamsters. More importantly, we have shown specific knockdown of transcripts encoding a cysteine protease, *Ov*-CB1 of *O. viverrini*. To our knowledge, this is not only the first description of genetic transformation of *O. viverrini*, but also the first report of successful RNAi in this parasite. Notably, key enzymes involved in the RNA interference machinery and pathways including dicer and drosha-like proteins have been detected in recent transcriptomic analyses of *O. viverrini* (Young et al., 2010).

RNAi targeting cathepsin B-like enzymes has been demonstrated in *S. mansoni* (Correnti, Brindley, and Pearce, 2005; Skelly, Da'dara, and Harn, 2003) and *Fasciola hepatica* (McGonigle et al., 2008). Cathepsin B1 of *O. viverrini* is an essential enzyme for digestion of host hemoglobin, a primary food source for this parasite, and may also play a role in pathogenesis due to irritation of the bile duct. In *O. viverrini*, at least two forms of cathepsin B are expressed including *Ov*-CB-1 and *Ov*-CB-2 (Sripa et al., 2010b). *Ov*-CB-1 is also secreted as an active zymogen that is capable of *trans*-activating the related enzyme *Ov*-CF-1. We have previously predicted that *Ov*-CB-1 regulates *Ov*-CF-1 activity and that both enzymes participate to degrade host tissue contributing to the development of liver fluke-associated cholangiocarcinoma (Sripa et al., 2010b). Accordingly, we plan to investigate the impact of RNAi targeting *Ov*-CB1 on the activity ascribable to *Ov*-CF-1. Peak suppression levels were seen at three days after exposure to the dsRNA, although strong suppression was still apparent at nine days after treatment. These results are redolent of those described with *S. mansoni* where the effect of knockdown of cathepsin B1 expression by RNAi remained evident for at least 30 days after

treatment (Correnti, Brindley, and Pearce, 2005). It will be of interest now to investigate RNAi targeting other genes of *O. viverrini* (and *C. sinensis*), including thioredoxin peroxidase, cathepsin F and other mediators that are predicted to play pivotal roles in molecular carcinogenesis (Smout et al., 2009; Suttiprapa et al., 2008; Suttiprapa et al., 2009). Whereas RNAi is efficient in the two other flukes reported to date, schistosomes and *Fasciola*, it is thought that some genes are more responsive than other to this manipulation, perhaps influenced by the degree of difficulty in delivering the dsRNA or siRNA to target organs or tissues within the flukes (Krautz-Peterson et al., 2010).

In overview, we introduced genetic material by electroporation into *O. viverrini*, demonstrating the feasibility of this route of transformation of this neglected tropical diseases trematode. Second, the findings with silencing of an endogenous gene, *Ov*-CB1, suggested the existence of a viable and functional RNAi pathway in this liver fluke. We consider that these findings will not only facilitate further investigation of gene function in *O. viverrini* by RNAi approaches, including investigation of novel intervention targets, but they may likewise provide a path forward for genetic manipulation of other, even less-studied, food-borne trematodes (Rinaldi et al., 2008; Sripa et al., 2010a).