

## Hepatitis D antigens cause growth retardation and brood-size reduction in *C. elegans*

Li-Wei Lee<sup>1</sup>, Tsui-Yun Chang<sup>3</sup>, Hsiao-Wen Lo<sup>2</sup>, Szecheng J. Lo<sup>1,2,3</sup>

<sup>1</sup>Department of Life Science, Chang Gung University, Tao-Yuan 333, Taiwan, <sup>2</sup>Graduate Institute of Biomedical Sciences, Chang Gung University, Tao-Yuan 333, Taiwan, <sup>3</sup>National Yang-Ming University, School of Life Science, Institute of Microbiology and Immunology, Taipei 112, Taiwan

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and Methods
  - 3.1. Plasmid construction
    - 3.1.1. pFib-LD-SS
    - 3.1.2. *pP<sub>myo-2</sub>::GFP::icr::DsRed::SD I*
    - 3.1.3. *pP<sub>myo-2</sub>::GFP::icr::DsRed::SD II and pP<sub>myo-2</sub>::GFP::icr::DsRed::SD III*
    - 3.1.4. *pP<sub>myo-2</sub>::GFP::icr::DsRed::SD441, SD327, SD267 and SD201*
    - 3.1.5. *pHsp16.4I::GFP::icr and pHsp16.4I::GFP::icr::DsRed::SD I*
    - 3.1.6. *L-HDAg RNAi construct and feeding experiment*
  - 3.2. Worm culture
  - 3.3. Microinjection and microscopy
4. Results
  - 4.1. Phenotypes of transgenic worms expressing hepatitis D antigens
  - 4.2. Mapping the domain of HDAg caused worm growth retardation and brood-size reduction
  - 4.3. Sensitivity of larval stage to HDAg effect
  - 4.4. The effect of various genotypes of HDAg on transgenic worms
5. Discussion
6. Acknowledgement
7. References

## 1. ABSTRACT

*Caenorhabditis elegans* is a model organism that has been used to study human bacterial and viral pathogenesis. We report here the expression of human hepatitis delta viral antigens (HDAg) in *C. elegans* and measure the effect on the sterility, growth, and brood size in transgenic worms. Expression of HDAg under two different promoters, *fib-1* (a ubiquitous promoter) and *myo-2* (a pharynx-specific promoter), was achieved in *C. elegans* using dicistronic or tricistronic vectors derived from the operon CEOP5428. Transgenic worms expressing HDAg ubiquitously resulted in 20% to 70% sterility while those expressing HDAg in the pharynx displayed 70% sterility. Most of worms expressing HDAg in pharynx were arrested at larvae stage 2 or 3 and displayed a 70% reduction in brood size. Domain mapping experiments suggested that the nuclear localization signal of HDAg is required for the observed effect. Heat-shock induction of HDAg expression revealed that L4 larvae were the most sensitive to brood size reduction. These studies demonstrate that *C. elegans* can provide an additional model for studying HDAg interactions with host targets.

## 2. INTRODUCTION

Hepatitis delta virus (HDV) was first discovered as a new antigen from patients infected with hepatitis B virus (HBV) in 1977 (1). The clinical importance of HDV is to induce the fulminant of hepatitis by either superinfection in HBV patients or coinfection with HBV. However, the underlying pathogenic mechanism of HDV is still unclear. Molecular biology studies have revealed that HDV contains a single-stranded, negative sense, circular RNA genome about 1700 nucleotides long, which is enveloped by HBV surface antigens (HBsAgs) (2). Although the propagation of HDV requires the help of HBV, the replication of the HDV genomic and antigenomic RNA is independent of HBV and is mediated through a “double rolling circle” mechanism without DNA intermediates (2, 3).

HDV encodes two proteins (HDAgs), the small (SDAg; 24 KDa) and large (LDAg; 27 KDa) isoforms, from the same open reading frame (4). SDAg is required for HDV genome replication via binding to the host DNA-dependent RNA polymerase II and redirecting the enzyme

to copy the RNA template (5). L<sub>DAg</sub> is produced during the late stage of RNA replication and is involved in an RNA editing mechanism to convert a UAG codon into UGG (6, 7). This modification changes the amber stop codon of S<sub>DAg</sub> to a tryptophan codon and results in an extension of 19 amino acid residues at the C-terminus. In contrast to S<sub>DAg</sub>, the function of L<sub>DAg</sub> is required for viral particle assembly, secretion and inhibition of HDV RNA replication (4, 8).

The functional domains of H<sub>DAg</sub> have been well characterized and contain a coil-coiled sequence (CCS, oligomerization domain) (9), RNA binding motif (RBD) (10), nuclear localization signal (NLS) (11) and RNA Polymerase II binding domain (5, 12). H<sub>DAg</sub> is able to form homodimers, heterodimers or oligomers by interaction between their coil-coiled domains (9). H<sub>DAg</sub> also binds to different RNA species, host transcriptional factors (13) and cellular nucleolar proteins such as B23 (14) and nucleolin (15). Interactions with these cellular factors have been suggested to enhance the viral RNA replication and to associate with the pathogenesis of HDV. Furthermore, H<sub>DAg</sub> is modified with several post-translational modifications (16), including phosphorylation (17, 18), acetylation (19), methylation (20), sumolization (21) and isoprenylation (8). These modifications reflect that H<sub>DAg</sub> reacts with host factors very actively and such virus-host interaction may be important in pathogenesis. To study HDV pathogenesis, transgenic mice have been generated but only mild or no pathogenic effect was observed (22). Cell-based systems are still the most widely used model to study the effect of HDV, however, these systems are only limited to detecting changes at the molecular and cellular level which are not easily observed by microscopy. We propose that *Caenorhabditis elegans* can be a suitable model to investigate HDV dependent effects on observable phenotypes.

*C. elegans* is a simple, free-living soil nematode with around 1000 cells in adults. It has been used as a model in many biological research fields, including cell biology, neuroscience and aging. In addition, it has been applied in the field of toxicology (for example, the effects of heavy metals Pb, Hg, Cd and Cr), human diseases (for example, Alzheimer's disease and Huntington's disease) and host-pathogen interaction (for example, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) (23-27). Recent studies of *C. elegans* as a viral pathogenesis model (28-30) prompted us to test whether H<sub>DAg</sub> could affect worm phenotypes. Here, we observed that expression of H<sub>DAg</sub> in *C. elegans* pharynx tissue resulted in growth retardation and brood size reduction. We have also observed that the nuclear localization signal (NLS) of H<sub>DAg</sub> plays an important role in promoting these phenotypes.

### 3. MATERIALS AND METHODS

#### 3.1. Plasmid construction

To introduce various lengths of HDV cDNA into *C. elegans*, several expression vectors were constructed,

mainly derived from the backbone of the CEOP5428 operon (31), which are designed to co-express marker proteins (green fluorescence protein; GFP or red fluorescence protein, RFP; mCherry or DsRed) and the HDV cDNA as described below. Some constructs were described previously (32).

#### 3.1.1. pFib-LD-SS

Plasmid pFib-LD-SS encodes two fusion proteins, nucleolar fibrillarin fused with GFP (FIB-1::GFP) and DsRed fused with large delta antigen (DsRedLD), and a small HBV surface antigen (sHBS). To construct this plasmid, an ICR-sHBS fragment was generated by PCR and cloned into plasmid Fib-LD (32) at the EcoRI site. First, the ICR fragment was generated by PCR using primers: forward primer ICR-F2 containing an EcoRI site (5'- aaagaag **gaat tct** cca aaa tca tgc tta cat ttt -3') and reverse primer ICR-R2 containing a NotI site (5'- cggtaga **gcggccgc** tac cct gta ata ata tat taa ac-3'). The sHBS gene was generated using primers: HBVs-F (5'- GGACCCT**GCGGCCGC**ATGGAGAACATCACATCAG GATTC-3') with a NotI site and HBVs-R (5'- GTTTTGT**GAATTCT**TAAATGTATACCCAAAGACAA AAG-3') with an EcoRI site. These two fragments were cut with NotI, ligated and amplified with primers ICR-F2 and HBVs-R to generate the ICR-sHBS fragment. This fragment was then cut with EcoRI and cloned into plasmid Fib-LD to make the pFib-LD-SS plasmid. All plasmids were verified by nucleotide sequencing.

#### 3.1.2. pP<sub>myo-2</sub>::GFP::icr::DsRed::SD I

pP<sub>myo-2</sub>::GFP-icr-DsRed::LD1 (32) was separately digested with BglII/SpeI and EcoRI/SpeI to collect two fragments, P<sub>myo-2</sub>::GFP-icr-DsRed and unc-54 3'UTR, individually. A cDNA fragment of S<sub>DAg</sub> from genotype I gene (SDI in short) was obtained by digested of pMOD3-DsRed::SDI (generated in this laboratory) with BglII/EcoRI. Finally, these three fragments were ligated to generate pP<sub>myo-2</sub>::GFP::icr::DsRed::SD I.

#### 3.1.3. pP<sub>myo-2</sub>::GFP::icr::DsRed::SD II and pP<sub>myo-2</sub>::GFP::icr::DsRed::SD III

The full length of both SD II and III were amplified from pN2-SD2 and pN2-SD3 (generated in this lab) using pairs of Primers 9 and 10, and 9 and 11, respectively (see Table 1). The PCR products were cut with BglII and XmaI and then used to replace SDI gene in pP<sub>myo-2</sub>::GFP::icr::DsRed::SDI.

#### 3.1.4. pP<sub>myo-2</sub>::GFP::icr::DsRed::SD441, SD327, SD267 and SD201

All deletion constructs were based on the cDNA sequence of HDV genotype I and the functional domain map shown in Fig.4A. SD441 contains the 441 bp cDNA fragment (147 amino acids) of S<sub>DAg</sub> which was obtained by PCR using primers 1 and 2, then cut with BglII and XmaI and replaced the full length of SDI gene in P<sub>myo-2</sub>::GFP::icr::DsRed::SDI. A similar strategy was used to construct SD327 (327 bp) by using Primer 1 and 3, SD267 (267 bp) by using Primer 1 and 4, and SD201 (201 bp) by using Primer 1 and 8. All primers are listed in Table 1.

**Table 1.** The primers used in this study

Number	Name	Sequences
1	F-BglII-SD	GCCACCACCTGTTCTCTGCTCAGAT
2	R-SD441stop-SmaI	TCACACCCCCGGGTACGGGCCGGCT
3	R-SD327stop-SmaI	ATAGCTGCTTCCCCGGGTCACTCGAGGGCC
4	R-SD267stop-SmaI	GTGAATCCCCCGGGTCAAGGCCTCTTC
5	F-BglIIstart-88SD	AAGAAAGAAGAGATCTATGCTCGAGAGAG
6	F-BglIIstart-202SD	GGATAAGGATAGATCTATGGCACCCCCGG
7	R-SD-XmaI	AAGCCGCCCCCGGGCGCTCCCCCTC
8	R-SD201stop-XmaI	TTCTTCGCCCCCGGGTCACCCCTCT
9	F-BglII-SD2&3	TCCGCTAGCCAGATCTATGAGCCAA
10	R-SD2-XmaI	TCGACTGCACCCGGGTACGGGA
11	R-SD3-XmaI	TCGACTGCACCCGGGTATGGGAA
12	F-DsRed	GACTACACCATCGTGGAGCAGTAC

### 3.1.5. pHsp16.41::GFP::icr and pHsp16.41::GFP::icr::DsRed::SD I

The heat shock promoter was isolated from Hsp16.41::NLSGFP (33) by cutting with HindIII and KpnI and replaced the myo-2 promoter of Pmyo-2::GFP::icr and Pmyo-2::GFP::icr::DsRed::SD I. To obtain the transgenic worms, a mixture of plasmids, including Hsp16.41::GFP::icr or Hsp16.41::GFP::icr::DsRed::SD I, Pmyo-2::hsf-1 and pRF-4, were co-injected into sy-441, a heat shock factor-1 mutant. For induction of SDAg by heat shock, transgenic worms carrying all three plasmids were incubated at 34°C for 1 hr and then recovered for 4 hrs at 20°C.

### 3.1.6. L-HDAg RNAi construct and feeding experiment

To construct the RNAi vector, LDAg gene fragment (650 bp) was purified from P<sub>fib-1</sub>-FIB-1::GFP-icr-DsRed::LD1 (32) by digesting with EcoRI and BglII and cloned into L4440. RNAi plates were prepared as follows: bacteria HT115 transformed with the L4440 or L4440-LDAg plasmid were induced by adding 1 mM of IPTG for 2 hrs and spread on plates supplemented with ampicillin (100 µg/mL), tetracyclin (25 µg/mL) and 1mM of IPTG. Plates were then incubated at room temperature for 2 days before use. For an RNAi feeding experiment, transgenic worms at the L1 or L2 stage were picked and put onto the plates with one worm per plate. The reproduction activity of a worm was scored every day. Data are averaged from at least three independent experiments. All statistical analyses were performed using Student's t test.

### 3.2. Worm culture

N2 (wild-type) was obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota). Worms were cultured in Nematode Growth Medium (NGM) following standard methods (34). For light and fluorescence microscopic observation, worms were paralyzed in one drop of 0.2% levamisole (Sigma, USA) in PBS and placed on a slide with a 5% agarose pad.

### 3.3. Microinjection and microscopy

Worm microinjection was performed by a previously described procedure (35) using a FemtoJet system (Eppendorf AG, Hamburg, Germany). N2 young adults were picked and placed on a 2% agarose pad on a

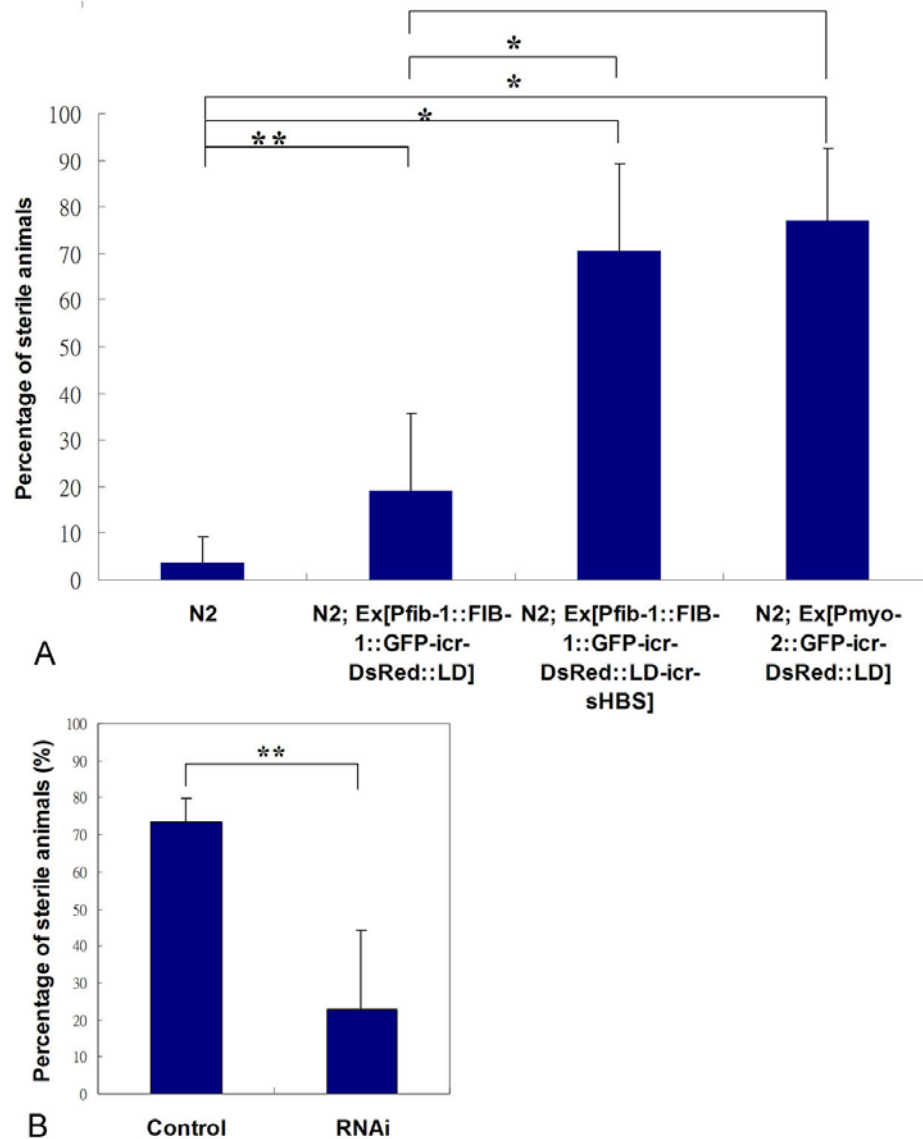
cover slide (24x50 mm) (Marienfeld laboratory glassware; Germany). The injection mixture contained plasmids generated in this study along with pRF-4, which was included as a screening marker. Images of transgenic worms were acquired using an Olympus IX71 and processed with a DPC controller (Olympus, Japan).

## 4. RESULTS

### 4.1. Phenotypes of transgenic worms expressing hepatitis D antigens

To test whether *C. elegans* can be used as a model to study HDV antigen interacting molecules, we initially tested several tissue-specific promoters to drive HDAg expression, including intestine (*ges-1*), neuron (*mec-7*), pharynx (*myo-2*) and whole body (*fib-1*). We observed that when HDAg was expressed in pharynx it caused most severe phenotypes. Here, we report results from worms transformed by tricistronic vectors (pFib-LD-SS) which can express fibrillarin fused with GFP (FIB-1::GFP), DsRed fused with large delta antigen (DsRedLD) and HBV surface antigen (SS) under the promoter of fibrillarin (*fib-1*). We also report on worms transformed by bicistronic vectors (pFib-LD) which expressed FIB-1::GFP and DsRedLD under the *fib-1* promoter. We first screened worms expressing GFP and DsRed under a fluorescence dissecting microscope and singled out each transgenic worm to a new plate to observe the fertility. As shown in Figure 1A, transgenic worms harboring bicistronic and tricistronic vectors displayed 20% and 70% of sterility relative to a wild-type control, respectively. Other transgenic worms harboring bicistronic vectors (pMyo-LD) which expressed GFP and DsRedLD under the *myo-2* promoter, a pharynx-specific promoter, showed 70% sterility similar to those bearing tricistronic vectors (Figure 1A).

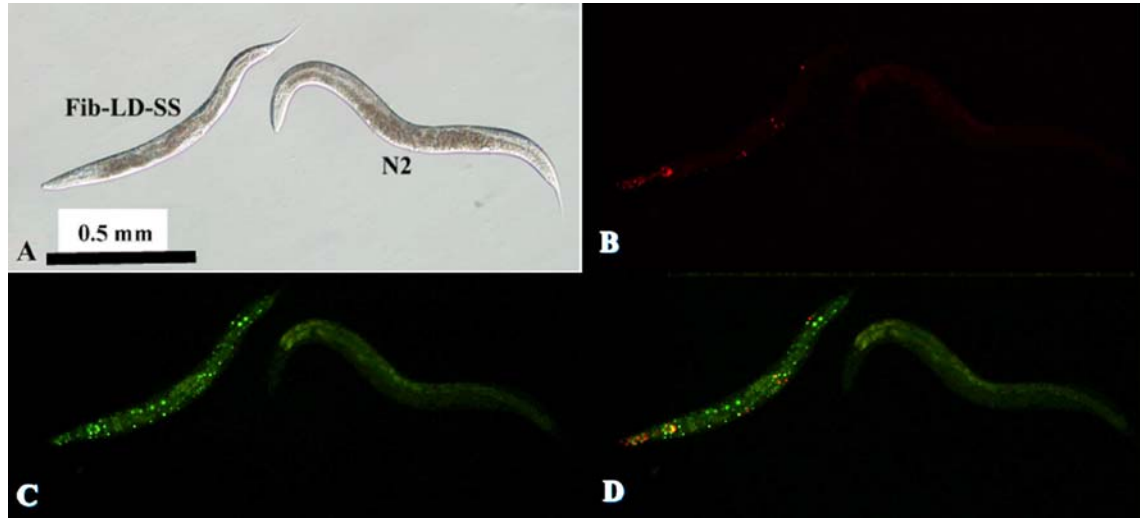
To examine whether the expression of DsRedLD in pharynx is the cause of the observed sterility phenotype, we performed RNAi experiments to feed pMyo-LD vector-containing transgenic worms with bacteria expressing a double-stranded RNA of LDAg, which showed no homology to *C. elegans* by WormBase BLAST search. Results showed a reduction in the loss of sterility in the experimental groups as compared with the mock-treated group (Figure 1B). These results demonstrated that expression of HDAg in the pharynx can cause sterility in worms.



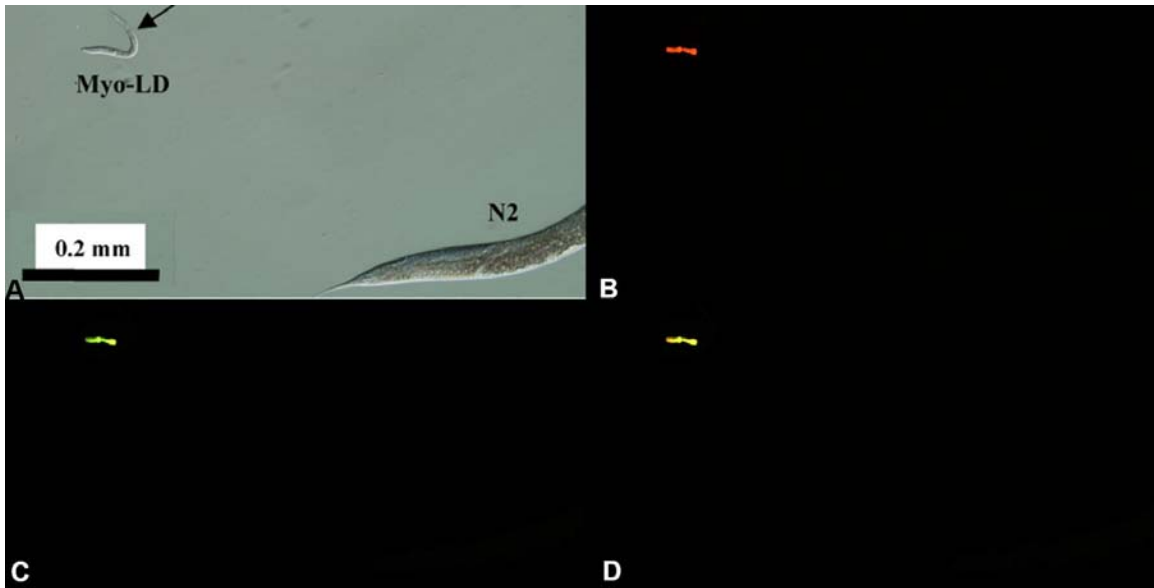
**Figure 1.** Sterility analysis of transgenic worms expressing hepatitis D viral antigens. (A) Plasmid Fib-LD-SS encodes three proteins, FIB-1::GFP fusion protein, DsRed::L-HDAg fusion protein and small HBV surface antigen, under the control of fib-1 promoter. These three genes are co-expressed and linked by the intercistronic region (icr) of OP. The sterile animals of three transgenic worms were counted from at least four individual experiments. The total numbers of animals used in the experiments are: N2, n=51; Fib-LD (N2;Ex[Pfib-1::FIB-1::GFP-icr-DsRed::LD]), n=61; Myo-LD (N2;Ex[Pmyo-2::GFP-icr-DsRed::LD]), n=67; Fib-LD-SS (N2;Ex[Pfib-1::FIB-1::GFP-icr-DsRed::LD-icr-sHBS]), n=56. Student's t test indicates that differences among the groups are significantly different (\*\*P < 0.05; \*P < 0.01); bar graphs correspond to mean  $\pm$  SEM (N= 4~6). (B) L-HDAg RNAi. Myo-LD transgenic worms (N2;Ex[Pmyo-2::GFP-icr-DsRed::LD]) were either fed with RNAi bacteria carrying L-HDAg gene (RNAi) or bacteria harboring L4440 plasmid only (Control). The worms fed with RNAi bacteria could partially rescue the sterility phenotype. The results are averaged from three individual experiments. Student's exact t test indicates that differences between two groups are significantly different (\*\*P < 0.05); bar graphs correspond to mean  $\pm$  SEM (N= 3).

While studying the sterility phenotype of transgenic worms, we observed that some worms displayed a growth retardation phenotype. We thus examined the growth rate of pFib-LD-SS and pMyo-LD transformed worms that displayed a higher rate of sterility and found tricistronic-bearing worms had a similar growth rate as non-transgenic wild-type worms (N2) (Figure 2). In

contrast, more than 50% of transgenic worms expressing DsRedLD in the pharyngeal tissue showed a smaller body size than N2 at day 5 after hatching (Figure 3). These two transgenic worms had a similar rate of sterility but displayed a varied body size, suggesting that HDAg expressed in different tissues may interact with different molecules to interfere with the growth pathway differently.



**Figure 2.** Comparison of body size of transgenic Fib-LD-SS and wild-type N2 worms at day 5 culture. Photographs were taken to include two worms under the same field. (A) Bright-field image. (B) Fluorescence microscopy with a filter for detection of DsRedLD. RFP can be detected in the transgenic worm body (labeled with Fib-LD-SS on the left side) but signal is very weak. (C) Fluorescence microscopy with a filter for detection of GFP. GFP is also observed within the whole body of worm but signal is as weak as RFP. (D) Merged view of DsRedLD (B) and GFP (C). Bar represents 0.5 mm.

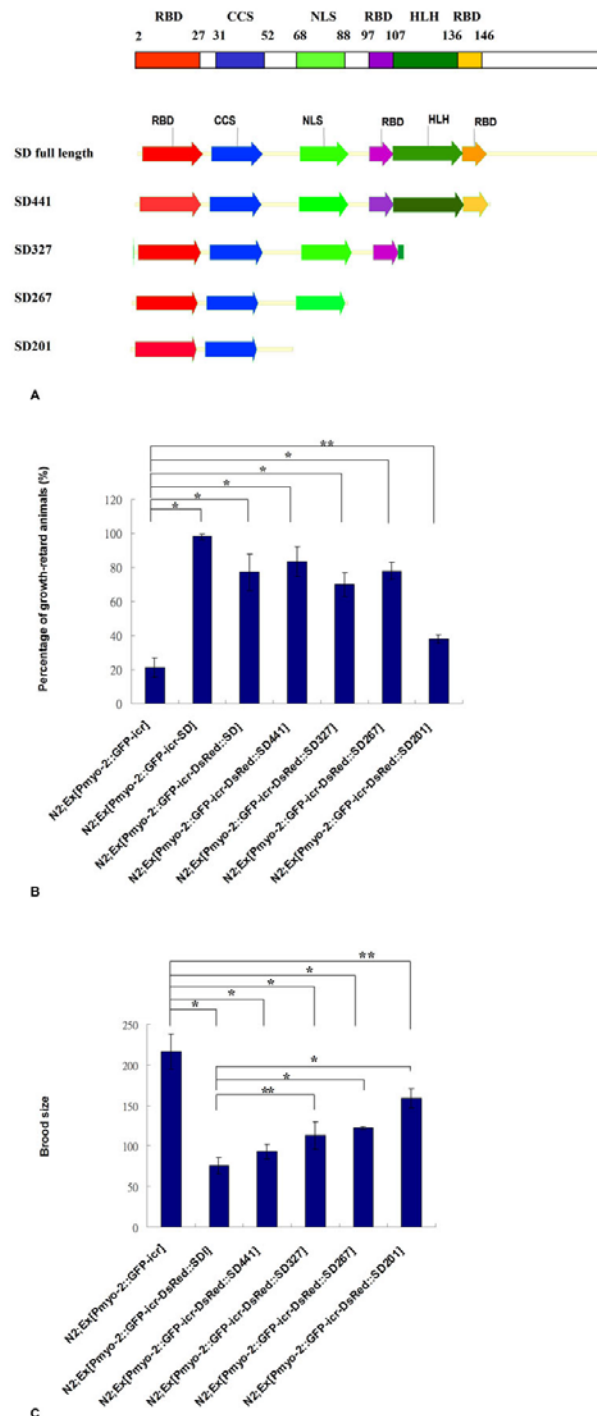


**Figure 3.** Comparison of body size of transgenic Myo-LD (upper left corner) and wild-type N2 (lower right corner) worms at day 5 in culture. Photographs were taken to include the whole Myo-LD transgenic worm but not N2. The body size is apparently much smaller for the Myo-LD worm compared to that of the wild-type strain (N2). (A) Bright-field image. (B) Fluorescence microscopy with a filter for detection of Ds-Red::LD. RFP was observed at the pharynx tissue of Myo-LD transgenic worm. (C) Fluorescence microscopy with a filter for detection of GFP. GFP was observed at the same region as RFP in the Myo-LD strain. (D) Merged view of Ds-Red-LD (B) and GFP (C). Bar represents 0.2 mm.

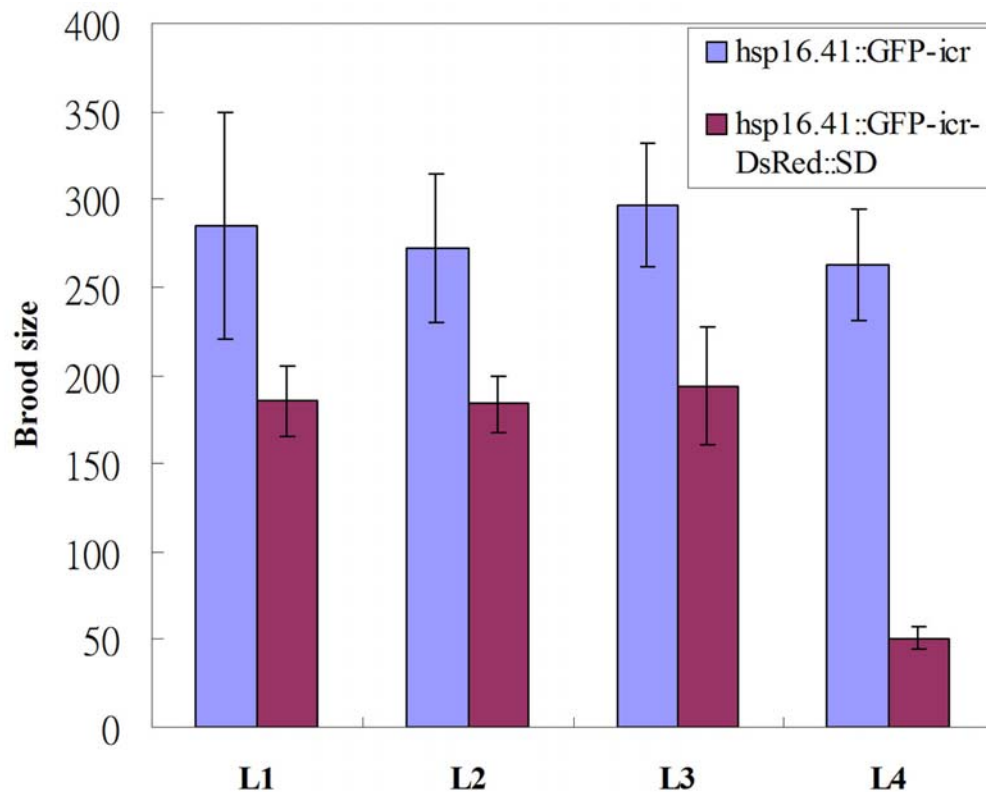
Our observation suggest that HDAG expression in the pharynx has severe effects on both sterility and growth rate.

Since it was easier and less-time consuming to do growth-rate analysis than to do the sterility analysis of transgenic worms, we focused on the effect of growth rate

in bicistronic vector transformed worms. Among a total of 244 DsRedSD transgenic worms analyzed, 98% of the animals were growth retarded and more than 50% were arrested at larval stage 2 (Figure 3). To distinguish if the effect could have resulted from DsRed expression alone or the HDAG, animals expressing an authentic form of SD instead of fusion protein of DsRedSD were generated.



**Figure 4.** Measurement of growth retardation and brood sizes of transgenic worms containing S-HDAg domain deletion constructs. (A) The partial plasmid map of P<sub>myo-2</sub>::GFP-icr-DsRed::SDI and S-HDAg domain deletion constructs. The location of each domain is shown above the scheme. Abbreviation: RBD, RNA-binding domain; CCS, coiled-coil sequence; NLS, nuclear-localization sequence; HLH, helix-loop-helix. (B) Growth retardation of transgenic worms. Except the N2;Ex[Pmyo-2::GFP-icr-DsRed::SD201] transgenic worms (\*\*P < 0.05), all other groups show a significant difference (\*P < 0.01) compared with N2;Ex[Pmyo-2::GFP-icr]. (C) Brood sizes of transgenic worms. The brood sizes of N2;Ex[Pmyo-2::GFP-icr-DsRed::SD201] and N2;Ex[Pmyo-2::GFP-icr-DsRed::SD267] showed a significant difference to N2;Ex[Pmyo-2::GFP-icr-DsRed::SDI] (\*P < 0.01). Data are averaged from three independent experiments. Student's t test indicates that differences between the groups are significantly different (\*P < 0.01; \*\*P < 0.05); bar graphs correspond to mean ± SEM (n = 3).



**Figure 5.** Effect of S-HDAg expression at the fourth larval stages. Plasmid Hsp16.41::GFP-icr is used as a control vector. Hsp16.41::GFP-icr-DsRed::SD encoded S-HDAg fused with DsRed which were expressed under Hsp16.41 promoter control. The measurement of brood sizes at different larval stages of transgenic worm (N2;Ex[hsp16.41::GFP-icr-DsRed::SD]) induced S-HDAg expression.

These animals showed an even more severe phenotype than those expressing DsRedSD (Figure 4B), demonstrating that the growth retardation is primarily due to HDAg and not from DsRed. Because a DsRed fusion protein was easier to detect under a fluorescence microscope, hereafter, DsRed fusion proteins were used to study the effects of various lengths of HDV or to investigate the consequence of expressing different HDV genotypes in worms.

#### 4.2. Mapping the domain of HDAg that induced worm growth retardation and brood-size reduction

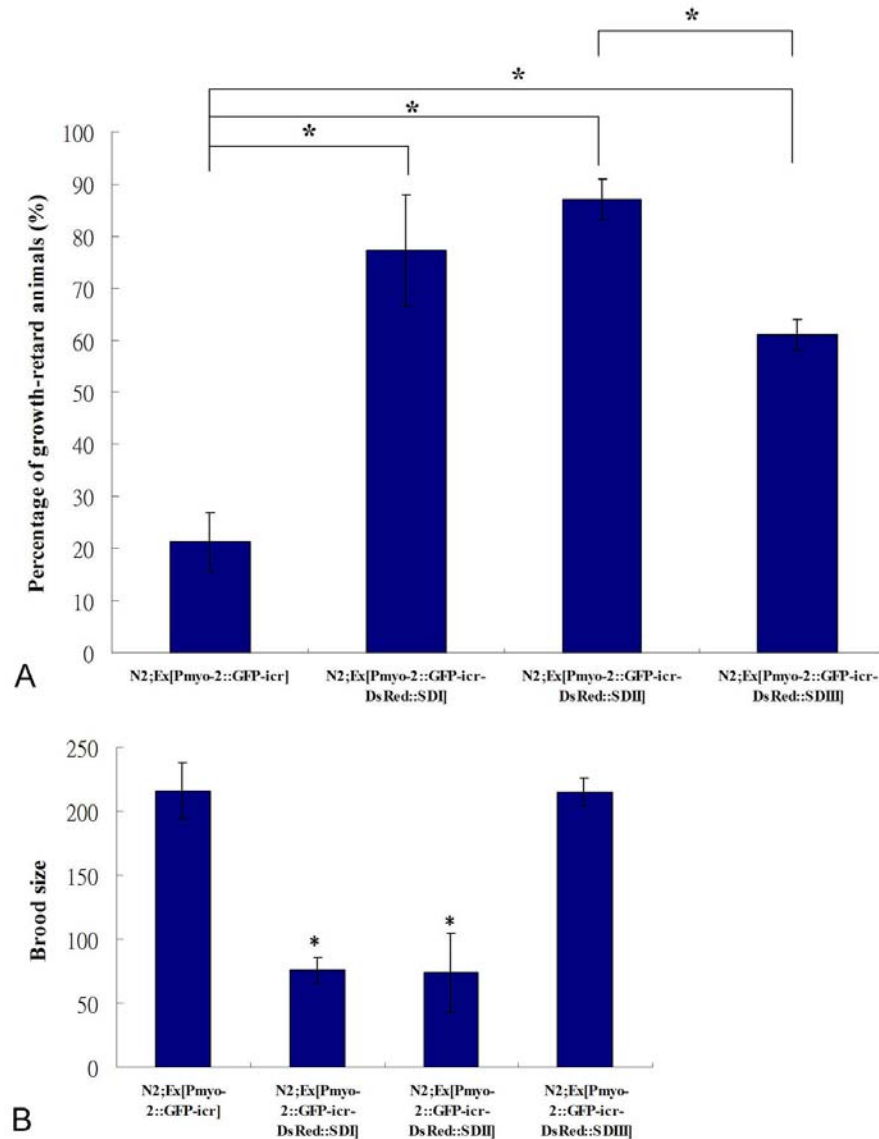
Several functional domains of HDAg have been determined, such as an RNA binding domain (RBD), a coil-coiled sequence (CCS) and a nuclear localization signal (NLS). We created different domain deletion mutants of DsRedSD (Figure 4A). Transgenic worms bearing full-length and various truncated forms of HDAg (SD441, SD327, SD267, and SD201) were subjected to the growth rate analysis. As shown in Figure 4B, the percentage of growth-retarded animals bearing various truncated forms of HDAg, SD441, SD327, and SD267, were similar to those bearing a full-length form of SD. In contrast, the rate of growth-retardation in worms bearing SD201 was reduced to only half as compared with all other transgenic worms (Figure 4B). When the brood size of each

group of transgenic worms bearing different lengths of SD were examined, we also found that the effect on brood size by expressing various versions of SD was consistent with the effect on growth (Figure 4C). The full-length SD bearing worms reduced brood size to 75 as compared with 210 of the control group. Those bearing SD441, SD327, and SD267 had an average brood size of 100, 110, and 120, respectively. In contrast, the SD201 transgenic worms had a brood size of 175. These results demonstrated that SD201 transgenic worms with the NLS deleted caused less effect on brood size and growth rate.

#### 4.3. Sensitivity of larval stage to HDAg effect

In order to narrow down when the HDAg might affect the pharyngeal muscle to result in a change in the brood size production, we generated transgenic worms bearing a vector which produces DsRedSD after raising the temperature to 25°C for an hour. We then analyzed the brood size of transgenic worms which were heat treated at various larval stages. As shown in Figure 5, the transgenic worms carrying a control plasmid have a similar brood size- 285, 272, 297 and 263 for L1, L2, L3 and L4, individually. However, induction of DsRedSD expression at L4 stage decreased the brood size of transgenic worms ( $51 \pm 7$ ) as compared with the brood size of 170 produced

by other transgenic worms which were heat treated at the



**Figure 6.** Effects of different HDV genotypes on growth retardation and brood sizes in transgenic worms. (A) Measurement of growth retardation of transgenic animals. Student's t test indicates that differences between the groups are significantly different (\* $P < 0.01$ ); bar graphs correspond to mean  $\pm$  SEM (N= 3). A statistically significant difference is also noted between genotype II and III (\* $P < 0.01$ ). (B) Statistically significant differences among transgenic worms with different genotypes are shown on the graph (\* $P < 0.01$ ). No significant difference is observed between control and genotype III. Data are averaged from three independent experiments.

L1, L2, or L3 stage (Figure 5). These results suggest that larvae stage 4 is sensitive to HDAG expression.

#### 4.4. The effect of various genotypes of HDAG on transgenic worms

Although 8 different clades of HDV have been identified worldwide, three genotypes of HDV are more representative to geographical distributions and found to cause variable liver diseases from mild to severe (36-38). In general, HDV genotype III is associated with more severe liver disease and type II is mild. The disease pattern of type I can range from severe to mild. To determine whether the

observed phenotypes in transgenic worms correlate with the disease pattern in humans, three transgenic worms bearing three genotypes of HDAG were generated. When the growth rate was analyzed, transgenic worms bearing genotype I and II of HDV displayed an 80% to 85% decrease in growth rate as compared with the control group (20%) while those bearing genotype III of HDV displayed 60% retardation in the growth rate (Fig.6A). When the brood was analyzed, those transgenic worms bearing genotype III HDV had a similar brood size (~250) as the control group while those worms bearing genotype I or II of HDV had about 75 (Figure 6B). The results of genotype



III effects do not correlate with human disease outcomes, which could be because HDV pathogenesis includes several steps and requires the presence of HBV.

### 5. DISCUSSION

This study is the first report to use *C. elegans* as a model to examine the possible pathological effect of HDV antigen and demonstrated that expression of both LDAg and SDAg could cause growth retardation and lower broods. Domain deletion analyses demonstrated that the nuclear localization signal (NLS) of HDAg is important for the observed phenotypes. Which worm nuclear proteins interact with HDAg to cause these effects remains unknown. Many cell-based models have demonstrated that HDAg can bind to a variety of host nuclear factors such as transcription factor YY-1 and its coactivators, CBP and p300 (13). Binding of YY-1 by HDAg then enhances the expression of nucleolar phosphoprotein B23, and interacting with B23 with HDAg could increase HDV RNA replication (14). HDAg has also been shown to selectively inhibit the host Pol II transcription (39) and associate with Pol I by binding with Pol I-associated transcription factor SL-1 (40). Although the target molecules interacting with HDAg inside nucleus are not identified in this study, HDAg interacting molecules presumably are important in regulating worm growth and brood production.

Two major signal pathways, the insulin-like and TGF-beta-like pathways, are well known in regulating worm development and growth (41, 42). The growth retarded worm phenotype in Figure 3 may arise through the interference of the TGF-beta signaling pathway. It has been reported that HDAg can interfere the TGF-beta signaling pathway by interacting with Smad3 (43) and the TGF-beta signaling pathway is known to be involved in the regulation of worm body size as demonstrated by analysis of *sma* mutants (41, 44). The observed growth retardation in the worms caused by HDAg is consistent with the reduction in the brood size, except for the genotype III HDAg which had no brood-size reduction effect but displayed 60% growth retardation. The mechanism and signal pathways involved in the brood-size reduction are relatively unclear because many unrelated treatments can result in brood size reduction, for example the exposure of heavy metals, irradiation, and genetic mutation (26, 41, 45). Nevertheless, one report indicates that the growth-rate and brood-size control could be through different pathways (42). Since many amino acids of HDAg are variable among three genotypes (46), it is very possible that they are commonly interacting with molecules in TGF-beta-like pathways but interact differently with molecules in brood-size control.

All transgenic worms generated in this study were in an array form to express HDAg, which makes it difficult for further biochemical manipulation to identify the HDAg interacting molecules directly. However two hints from this study can be used to search for the molecules which are responsible for brood size control in the future; one from the domain-mapping experiment (Figure 4) and the other from temperature-sensitive study (Figure 5). The former results indicated the minimum

sequence of HDAg for the effect contains two functional domains, RNA-binding domain and coil-coiled domain (Figure 4A), suggesting the interacting molecules could be RNA or coil-coiled domain-containing proteins. To verify this assumption, immunoprecipitation analyses using FLAG tagged HDAg may help to identify the targets. The later results suggest that the putative molecules interacting with HDAg either appear only in the L4 stage or play a vital role in pharynx at the L4 stage. As compared to the mouse transgenic model and cell-based systems, *C. elegans* has the advantage of providing easily scored phenotypes which can lead to identification of HDAg interacting molecules and may be useful to explore mechanisms of HDV pathogenesis. However, this advantage relies on the hypothesis that HDAg interacting molecules are evolutionarily conserved from worms to humans.

### 6. ACKNOWLEDGEMENT

We would like to thank Scott C. Schuyler (Chang Gung University) for critical comments on the manuscript. Thanks are due to members of Lo lab for helpful discussion, S. Shaham (Rockefeller University) for providing plasmids, and CGC (Caenorhabditis Genetics Center) for providing different strains of worms. This work was supported by grants from the Ministry of Education (EMRPD170131), the National Science Council (NSC 96-2320-B-182-002-MY3), and the National Health Research Institute (NHRI-EX96-9521) to S.J.L.

### 7. REFERENCES

1. M. Rizzetto: Hepatitis D: thirty years after. *J Hepatol*, 50(5), 1043-50 (2009)
2. J. M. Taylor: Hepatitis delta virus. *Virology*, 344(1), 71-6 (2006)
3. M. Chao: RNA recombination in hepatitis delta virus: implications regarding the abilities of mammalian RNA polymerases. *Virus Res*, 127(2), 208-15 (2007)
4. M. M. Lai: RNA replication without RNA-dependent RNA polymerase: surprises from hepatitis delta virus. *J Virol*, 79(13), 7951-8 (2005)
5. Y. Yamaguchi, T. Mura, S. Chanarat, S. Okamoto and H. Handa: Hepatitis delta antigen binds to the clamp of RNA polymerase II and affects transcriptional fidelity. *Genes Cells*, 12(7), 863-75 (2007)
6. S. Sato, S. K. Wong and D. W. Lazinski: Hepatitis delta virus minimal substrates competent for editing by ADAR1 and ADAR2. *J Virol*, 75(18), 8547-55 (2001)
7. J. L. Casey: RNA editing in hepatitis delta virus. *Curr Top Microbiol Immunol*, 307, 67-89 (2006)
8. S. B. Hwang and M. M. Lai: Isoprenylation masks a conformational epitope and enhances trans-dominant inhibitory function of the large hepatitis delta antigen. *J Virol*, 68(5), 2958-64 (1994)

9. Y. P. Xia and M. M. Lai: Oligomerization of hepatitis delta antigen is required for both the trans-activating and trans-dominant inhibitory activities of the delta antigen. *J Virol*, 66(11), 6641-8 (1992)
10. C. Z. Lee, J. H. Lin, M. Chao, K. McKnight and M. M. Lai: RNA-binding activity of hepatitis delta antigen involves two arginine-rich motifs and is required for hepatitis delta virus RNA replication. *J Virol*, 67(4), 2221-7 (1993)
11. Y. P. Xia, C. T. Yeh, J. H. Ou and M. M. Lai: Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex. *J Virol*, 66(2), 914-21 (1992)
12. Y. Yamaguchi, J. Filipovska, K. Yano, A. Furuya, N. Inukai, T. Narita, T. Wada, S. Sugimoto, M. M. Konarska and H. Handa: Stimulation of RNA polymerase II elongation by hepatitis delta antigen. *Science*, 293(5527), 124-7 (2001)
13. W. H. Huang, R. T. Mai and Y. H. Lee: Transcription factor YY1 and its associated acetyltransferases CBP and p300 interact with hepatitis delta antigens and modulate hepatitis delta virus RNA replication. *J Virol*, 82(15), 7313-24 (2008)
14. W. H. Huang, B. Y. Yung, W. J. Syu and Y. H. Lee: The nucleolar phosphoprotein B23 interacts with hepatitis delta antigens and modulates the hepatitis delta virus RNA replication. *J Biol Chem*, 276(27), 25166-75 (2001)
15. C. H. Lee, S. C. Chang, C. J. Chen and M. F. Chang: The nucleolin binding activity of hepatitis delta antigen is associated with nucleolus targeting. *J Biol Chem*, 273(13), 7650-6 (1998)
16. W. H. Huang, C. W. Chen, H. L. Wu and P. J. Chen: Post-translational modification of delta antigen of hepatitis D virus. *Curr Top Microbiol Immunol*, 307, 91-112 (2006)
17. J. J. Mu, H. L. Wu, B. L. Chiang, R. P. Chang, D. S. Chen and P. J. Chen: Characterization of the phosphorylated forms and the phosphorylated residues of hepatitis delta virus delta antigens. *J Virol*, 73(12), 10540-5 (1999)
18. C. W. Chen, Y. G. Tsay, H. L. Wu, C. H. Lee, D. S. Chen and P. J. Chen: The double-stranded RNA-activated kinase, PKR, can phosphorylate hepatitis D virus small delta antigen at functional serine and threonine residues. *J Biol Chem*, 277(36), 33058-67 (2002)
19. J. J. Mu, Y. G. Tsay, L. J. Juan, T. F. Fu, W. H. Huang, D. S. Chen and P. J. Chen: The small delta antigen of hepatitis delta virus is an acetylated protein and acetylation of lysine 72 may influence its cellular localization and viral RNA synthesis. *Virology*, 319(1), 60-70 (2004)
20. Y. J. Li, M. R. Stallcup and M. M. Lai: Hepatitis delta virus antigen is methylated at arginine residues, and methylation regulates subcellular localization and RNA replication. *J Virol*, 78(23), 13325-34 (2004)
21. C. H. Tseng, T. S. Cheng, C. Y. Shu, K. S. Jeng and M. M. Lai: Modification of small hepatitis delta virus antigen by SUMO protein. *J Virol*, 84(2), 918-27 (2010)
22. S. Guilhot, S. N. Huang, Y. P. Xia, N. La Monica, M. M. Lai and F. V. Chisari: Expression of the hepatitis delta virus large and small antigens in transgenic mice. *J Virol*, 68(2), 1052-8 (1994)
23. G. A. Silverman, C. J. Luke, S. R. Bhatia, O. S. Long, A. C. Vetica, D. H. Perlmutter and S. C. Pak: Modeling molecular and cellular aspects of human disease using the nematode *Caenorhabditis elegans*. *Pediatr Res*, 65(1), 10-8 (2009)
24. M. Hertweck, T. Hoppe and R. Baumeister: *C. elegans*, a model for aging with high-throughput capacity. *Exp Gerontol*, 38(3), 345-6 (2003)
25. A. Olsen, M. C. Vantipalli and G. J. Lithgow: Using *Caenorhabditis elegans* as a model for aging and age-related diseases. *Ann N Y Acad Sci*, 1067, 120-8 (2006)
26. Y. Guo, Y. Yang and D. Wang: Induction of reproductive deficits in nematode *Caenorhabditis elegans* exposed to metals at different developmental stages. *Reprod Toxicol*, 28(1), 90-5 (2009)
27. T. Komura, C. Yasui, H. Miyamoto and Y. Nishikawa: *Caenorhabditis elegans* as an alternative model host for legionella pneumophila, and protective effects of *Bifidobacterium infantis*. *Appl Environ Microbiol*, 76(12), 4105-8 (2010)
28. R. Lu, M. Maduro, F. Li, H. W. Li, G. Broitman-Maduro, W. X. Li and S. W. Ding: Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature*, 436(7053), 1040-3 (2005)
29. W. H. Liu, Y. L. Lin, J. P. Wang, W. Liou, R. F. Hou, Y. C. Wu and C. L. Liao: Restriction of vaccinia virus replication by a ced-3 and ced-4-dependent pathway in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A*, 103(11), 4174-9 (2006)
30. C. Wilkins, R. Dishongh, S. C. Moore, M. A. Whitt, M. Chow and K. Machaca: RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature*, 436(7053), 1044-7 (2005)
31. H. Pi, L. W. Lee and S. J. Lo: New insights into polycistronic transcripts in eukaryotes. *Chang Gung Med J*, 32(5), 494-8 (2009)
32. L. W. Lee, H. W. Lo and S. J. Lo: Vectors for co-expression of two genes in *Caenorhabditis elegans*. *Gene*, 455(1-2), 16-21 (2010)
33. T. Bacaj and S. Shaham: Temporal control of cell-specific transgene expression in *Caenorhabditis elegans*. *Genetics*, 176(4), 2651-5 (2007)

34. S. Brenner: The genetics of *Caenorhabditis elegans*. Genetics, 77(1), 71-94 (1974)
35. C. C. Mello, J. M. Kramer, D. Stinchcomb and V. Ambros: Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. Embo J, 10(12), 3959-70 (1991)
36. A. O. Shakil, S. Hadziyannis, J. H. Hoofnagle, A. M. Di Bisceglie, J. L. Gerin and J. L. Casey: Geographic distribution and genetic variability of hepatitis delta virus genotype I. Virology, 234(1), 160-7 (1997)
37. J. L. Casey, T. L. Brown, E. J. Colan, F. S. Wignall and J. L. Gerin: A genotype of hepatitis D virus that occurs in northern South America. Proc Natl Acad Sci U S A, 90(19), 9016-20 (1993)
38. J. C. Wu: Functional and clinical significance of hepatitis D virus genotype II infection. Curr Top Microbiol Immunol, 307, 173-86 (2006)
39. K. Lo, G. T. Sheu and M. M. Lai: Inhibition of Cellular RNA polymerase II transcription by delta antigen of hepatitis delta virus. Virology, 247(2), 178-88 (1998)
40. Y. J. Li, T. Macnaughton, L. Gao and M. M. Lai: RNA-templated replication of hepatitis delta virus: genomic and antigenomic RNAs associate with different nuclear bodies. J Virol, 80(13), 6478-86 (2006)
41. N. Watanabe, T. Ishihara and Y. Ohshima: Mutants carrying two sma mutations are super small in the nematode *C. elegans*. Genes Cells, 12(5), 603-9 (2007)
42. S. Luo, W. M. Shaw, J. Ashraf and C. T. Murphy: TGF-beta Sma/Mab signaling mutations uncouple reproductive aging from somatic aging. PLoS Genet, 5(12), e1000789 (2009)
43. S. H. Choi, S. H. Jeong and S. B. Hwang: Large hepatitis delta antigen modulates transforming growth factor-beta signaling cascades: implication of hepatitis delta virus-induced liver fibrosis. Gastroenterology, 132(1), 343-57 (2007)
44. Y. Nakano, Y. Nagamatsu and Y. Ohshima: cGMP and a germ-line signal control body size in *C. elegans* through cGMP-dependent protein kinase EGL-4. Genes Cells, 9(9), 773-9 (2004)
45. X. Liu, K. Cai, H. Feng, H. Yuan, M. Kong, L. Wu, Y. Wu and Z. Yu: Effects induced by keV low-energy ion irradiation in the nematode *Caenorhabditis elegans*. Radiat Environ Biophys, 46(3), 255-61 (2007)
46. C. R. Huang and S. J. Lo: Evolution and diversity of the human hepatitis D virus genome. Adv Bioinformatics, 323654 (2010)

**Key Words:** HDV, delta antigens, *C. elegans*, Transgenic Animals, Brood Size

**Send correspondence to:** Szecheng J. Lo, 259 Wen-Hwa 1<sup>st</sup> Road, Department of Biomedical Sciences, Chang Gung University, TaoYuan, Taiwan 333, Tel:88632118800x3295, Fax: 88632118392, E-mail: losj@mail.cgu.edu.tw

<http://www.bioscience.org/current/vol3E.htm>