Expression of hepatitis B virus surface antigens induces defective gonad phenotypes in Caenorhabditis elegans

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Abstract

AIM
To test whether a simple animal, Caenorhabditis elegans (C. elegans), can be used as an alternative model to study the interaction between hepatitis B virus antigens (HBsAg) and host factors.

METHODS
Three plasmids that were able to express the large, middle and small forms of HBsAgs (LHBsAg, MHBsAg, and SHBsAg, respectively) driven by a ubiquitous promoter (fib-1) and three that were able to express SHBsAg driven by different tissue-specific promoters were constructed and microinjected into worms. The brood size, egg-laying rate, and gonad development of transgenic worms were analyzed using microscopy. Levels of mRNA related to endoplasmic reticulum stress, enpl-1, hsp-4, pdi-3 and xbp-1, were determined using reverse transcription polymerase reaction (RT-PCRs) in three lines of transgenic worms and dithiothreitol (DTT)-treated wild-type worms.

RESULTS
Severe defects in egg-laying, decreases in brood size, and gonad retardation were observed in transgenic worms expressing SHBsAg whereas moderate defects were observed in transgenic worms expressing LHBsAg and MHBsAg. RT-PCR analysis revealed that enpl-1, hsp-4 and pdi-3 transcripts were significantly elevated in worms expressing LHBsAg and MHBsAg and in wild-type worms.
type worms pretreated with DTT. By contrast, only pdh-3 was increased in worms expressing SHBsAg. To further determine which tissue expressing SHBsAg could induce gonad retardation, we substituted the fbd-1 promoter with three tissue-specific promoters (myo-2 for the pharynx, est-1 for the intestines and mec-7 for the neurons) and generated corresponding transgenic animals. Moderate defective phenotypes were observed in worms expressing SHBsAg in the pharynx and intestines but not in worms expressing SHBsAg in the neurons, suggesting that the secreted SHBsAg may trigger a cross-talk signal between the digestive track and the gonad resulting in defective phenotypes.

CONCLUSION
Ectopic expression of three forms of HBsAg that causes recognizable phenotypes in transgenic worms suggests that C. elegans can be used as an alternative model for studying virus-host interactions because the resulting phenotype is easily detected through microscopy.

Key words: Hepatitis B virus; Caenorhabditis elegans; Green fluorescence proteins; Endoplasmic reticulum stress; Gonad retardation; Surface antigens

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Core tip: In the past, mouse and cell culture models have been used for studying the effects of hepatitis B virus antigens (HBsAg) on hosts. Both models have advantages and disadvantages in terms of economic and time concerns. In this study, we provide an alternative animal model, Caenorhabditis elegans (C. elegans), to demonstrate that SHBsAg can induce observable phenotypes which has never been reported in mouse and cell culture models. We suggest that C. elegans can serve as a new platform for studying various viral pathogenesis.

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INTRODUCTION
Human hepatitis B virus (HBV), a member of the family Hepadnaviridae, is a partially double-stranded DNA virus. The genome of HBV contains approximately 3200 nucleotides that encodes four open reading frames, namely surface (S), core (C), polymerase (P) and X in an overlapping but frame-shifted manner[1,3]. Infection with HBV induces a broad range of clinical outcomes, from asymptomatic hepatitis to fulminant hepatitis. Chronic hepatitis B carriers are highly associated with the development of liver cirrhosis and hepatocellular carcinoma[4,5]. Molecular biology analyses from tumor samples have revealed that HBV DNA integration could activate genes associated with the cell cycles, leading to abnormal cell proliferation[6,7]. Pathogenesis and etiology studies have found that X and truncated preS proteins play oncogenic roles[8-10].

Woodchucks were the first animal model for studying liver carcinogenesis caused by natural woodchuck hepatitis virus (WHV) infection[11,12]. A high incidence of liver tumor formation occurs in newborn woodchucks infected with WHV. Molecular dissection has revealed that c-myc oncogene level are highly elevated in liver tumors[13,14]. Later, the transgenic mouse model was applied to express an individual viral protein, such as the large hepatitis B surface antigen (LHBsAg) and X protein, which are driven by the albumin promoter for specific expression in the liver; to study the mechanisms of liver carcinogenesis induced by viral proteins[15,16]. In combining molecular biology analyses of the HBV X (HBx) gene in transfected cells, numerous studies have elucidated that the X protein is multifunctional and induces transactivation activity, signal transduction and cell death[9,17,18]. Recently, Geng et al[19] employed Caenorhabditis elegans (C. elegans), a soil nematode, to express HBx under a heat shock control and found that HBx induced cell apoptosis and necrosis through the interaction of HBx and CED-9, a human homolog of Bcl-2.

C. elegans was first used as a model organism for studying development and the nervous system because the species is transparent throughout its life span and in its adult form, possesses approximately 300 neurons out of 1000 somatic cells[20,21]. Because of its short-life cycle, simplicity, numerous available mutated forms, and ease of handling for knocking down specific gene, C. elegans is now a model for studying various biological topics, such as aging, human diseases, host-pathogen interaction and viral pathogenesis[22-25]. Because of a high percentage of genes in numerous cellular pathways is conserved across nematodes to vertebrates, a study of PEG-mediated Poxviridae infection in C. elegans revealed that the core genes of apoptosis (ced-3 and ced-4) control vaccinia virus replication in worms[26,27]. Therefore, C. elegans could serve as a new platform for virologists to study virus-host interaction and pathogenesis in addition to the currently used cell culture and mammalian models. In this study, we expressed three forms of HBsAg in C. elegans to determine different degrees of defects in gonad development.

MATERIALS AND METHODS

Plasmid constructions
Pα 1::gfp::icr::SHBsAg: A 1.5 kb fragment excised from the Pα 1::gfp::LD plasmid[28] by cutting with HindIII and Age I and was isolated and then inserted into the HindIII and Age I sites of pPD95.75 to generate Pα 1::gfp. Pα 1::gfp was then cut with EcoRI I and ligated with a 0.8 kb of icr::SHBsAg fragment which was isolated from Pα 1::LD::icr::SHBsAg to generate a 6.8 kb of Pα 1::
HBVs(M)+Not
HBVs(L)+Not

The plasmid was generated by substitution of the fib-1 promoter of PHb1-gfp::icr::SHBsAg with the mec-7 promoter which was isolated from Pmyo-2::gfp::icr::LD[28] to create a 6.3 kb of PHb1-gfp::icr::SHBsAg. Transgenic worms carrying this plasmid expressed both GFP and SHBsAg in neurons.

The plasmid was generated by substitution of the fib-1 promoter of PHb1-gfp::icr::SHBsAg with the ges-1 promoter which was isolated from Pges-1::gfp::icr::LD[28] to create a 6.6 kb of PHb1-gfp::icr::SHBsAg. Transgenic worms carrying this plasmid expressed GFP and SHBsAg in intestinal cells.

For transgenic worms, the presence of the transgene was confirmed by PCR using PA-3 primer pairs. For RT-PCR analysis, the following primer pairs were used: linker-F: 5'-aat tcaaaaagcggccgcagatctgtcgacatgcatgagctc-3'; linker-R: 5'-gttcgtgctgcctgctcctgtcgacatgcatgagctc-3'; HBVs(L)-NotI-F: 5'-gggaaagaaggggagagct-3'; HBVs(L)-SalI-R: 5'-gttcggtcagctgctcctgtcgacatgcatgagctc-3'; HBVs(M)+NotI-F: 5'-acctacatgctgcgctgcctgtcgacatgcatgagctc-3'; HBVs+SalI-R: 5'-gttcgtgctgcctgctcctgtcgacatgcatgagctc-3';
eft-2-F: 5'-gtggtgctaatccatgctccacac-3'; eft-2-R: 5'-tcc tccgtaaaacgtgtcctcct-3'; endoplasmin-F: 5'-gaat cctccaatgacaca-3'; endoplasmin-R: 5'-gcatctccctgg agcgcattcg-3'; hsp-4-F: 5'-ttctgaggtctgctcctgtcgacatgcatgagctc-3'; hsp-4-R: 5'-tctcggatttttgcctgctcctgtcgacatgcatgagctc-3'; PDI-F: 5'-gcggctccatacaagga-3'; PDI-R: 5'-ccctctgccagcactgta-3'; xbp-1-F: 5'-ctctgctgtcagaaagagcgcctgctcctgtcctgctcctgtcgacatgcatgagctc-3'; xbp-1-R: 5'-catgataaatgtatcactccatcgtc-3'.

Worm strains and culture
N2 (wild-type) worm was obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota) and cultured on Nematode Growth Medium (NGM) following standard methods[29]. Images of transgenic worms were acquired using Leica DM2500 equipped with CoolSNAP K4 (photomicroscopy) and processed with a MetaMorph (version 6.1).

Microinjection
Plasmid DNA was prepared by using QIAprep spin miniprep kit and the concentration was adjusted at 100 ng/μL in injection buffer (20 mmol/L potassium phosphate, pH 7.5, 3 mmol/L potassium citrate, pH 7.5, 2% polyethylene glycol, M.W. 6000). The injection mixture also contained pRF-4 which was included as a screening marker. Worm was placed onto 2% agarose pads and injected by capillary needle loaded with DNA mixture using a FemtoJet system (Eppendorf AG, Hamburg, Germany). The glass capillaries were purchased from World Precision Instruments (Kwik-FilC, borosilicate 16 glass capillaries, item number 1B100F-6, United States) and pull by Flaming/Brown micropipette puller (MODEL P-97, Sutter Instrument Co., United States).

Measurement of egg-laying activity and brood size
Worms were first synchronized and placed one worm per a single plate. The offspring in each plate were counted every day.

Microscopy
For visualization of GFP expression in transgenic worms, an upright fluorescence microscope (Leica DM2500) was used. For visualization of gonad structure and development a differential interference contrast (DIC) microscope was used and images were captured using a cool CCD (CoolSNAP K4).

Reverse transcription
The total RNA was extracted from transgenic worms expressed both GFP and HBsAg with TRIzol reagent. The reverse transcription reaction was first carried out with 4 μg of RNA, 2 μL of dNTP (10 mmol/L), 2 μL of Oligo-dT (10 mmol/L), and added DEPC H2O to 12 μL. After incubated at 68 °C for 5 min, the mixture was then added 4 μL of 5 × first-strand buffer (invertrogen), 2 μL of DTT (0.1 mmol/L, invertrogen), 1 μL of RNase inhibitor (invertrogen) and 1 μL of Reverse Transcriptase (invertrogen) and incubated at 42 °C for 50 min, and then 70 °C for

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15 min. The primers used in PCR analyses were listed as above.

RESULTS
Expression of three forms of HBsAg reduces egg-laying capability
To determine whether C. elegans can be a new platform for studying virus-host interaction we ectopically expressed three lengths of HBsAg (SHBsAg, MHBsAg, and LHBsAg) in worms under the control of the ubiquitous promoter fibrillarin (fib-1). Three HBsAg gene sequences were individually placed in a bicistronic vector behind a reporter gene, green fluorescence protein (GFP), which was used as a selection marker[30]. Transgenic worms were selected for the expression of GFP (Figure 1A-F) and maintained to characterize phenotypes. After synchronization, transgenic worms were singled out and placed on single plates, and the numbers of eggs produced by individual worms were counted every day. The results showed different egg-laying averages in a total of 60 transgenic animals in three groups expressing SHBsAg, MHBsAg, and LHBsAg (Figure 1G). On the fourth day after hatching, the wild-type worms (N2) displayed an egg-laying rate of 100% whereas those worms expressing SHBsAg, MHBsAg, and LHBsAg demonstrated laying-egg rates of approximately 8.6%, 10%, and 53.3%, respectively. Although the egg-laying rates of the three lines of transgenic worms increased in the following days, the maximum egg-laying rate was 41.1% for worms expressing SHBsAg, 70% for worms expressing MHBsAg and 81.7% for worms expressing LHBsAg at 7 d post-hatching (Figure 1B). The reduced rate of egg-laying in the three lines of HBsAg-expressing

Figure 1 Expression of various lengths of hepatitis B virus antigens in whole worms induced defects in the rate of egg-laying. A-F: Micrographs of transgenic worms expressing LHBsAg (A and B), MHBsAg (C and D), and SHBsAg (E and F) were captured under a bright-field microscope (A, C, and E) and a fluorescence microscope (B, D, and F). The heads of the worms are shown toward the left. The scale bar indicates 200 μm. G: Egg-laying capability of three lines of transgenic worms and wild-type worms (N2) shown using various color bars. The rate of egg-laying in 3 to 7 d post-hatching is shown above the bar. HBsAgs: Hepatitis B virus antigens.
worms was unlikely to have caused by the ectopic expression of GFP because worms carrying a plasmid with the sole function of expressing GFP throughout the body exhibited egg-laying capability of 100% (Table 1).

### Expression of SHBsAg causes the most severe gonad retardation

To understand why the expression of various lengths of HBsAg in transgenic worms caused a reduction in egg-laying capability, we examined the gonad development of the three types of transgenic worms under a DIC microscope. As shown in Figure 2 (upper two rows), at 52 h after hatching, wild-type worms had nearly completed the gonad development; by contrast, worms expressing SHBsAg exhibited a dramatic retardation of gonad development at 52 h post-hatching expect the worm expressing SHBsAg is at 72 h post-hatching. The gonad contour is indicated with dotted lines and the tip (distal end) is marked by asterisk.

### Table 1  Comparison of egg-laying ability and brood size among various transgenic worms

| Construct | Strain | Ecotopic proteins | Protein expression site | Egg-laying ability (%) | Brood size |
|-----------|--------|-------------------|-------------------------|------------------------|------------|
| Pfib-1::gfp::icr | N2 | GFP | Whole worm | 100 (n = 17) | 290 ± 15 (n = 17) |
| Pfib-1::gfp::icr::SHBsAg | N2 | GFP, SHBsAg | Whole worm | 9 (n = 60) | 66 ± 15 (n = 14) |
| Pfib-1::gfp::icr::MHBsAg | N2 | GFP, MHBsAg | Whole worm | 10 (n = 60) | 175 ± 50 (n = 15) |
| Pfib-1::gfp::icr::LHBsAg | N2 | GFP, LHBsAg | Whole worm | 54 (n = 60) | 239 ± 14 (n = 15) |
| Pmyo-2::gfp::icr::SHBsAg | N2 | GFP, SHBsAg | Pharynx | 83 (n = 60) | 163 ± 20 (n = 26) |
| Pges-1::gfp::icr::SHBsAg | N2 | GFP, SHBsAg | Intestine | 97 (n = 60) | 203 ± 50 (n = 32) |
| Pmec-7::gfp::icr::SHBsAg | N2 | GFP, SHBsAg | Neuron | 100 (n = 60) | 270 ± 42 (n = 26) |

HBsAgs: Hepatitis B virus antigens; GFP: Green fluorescence proteins; SHBsAg: Human hepatitis B virus small surface antigens; MHBsAg: Human hepatitis B virus middle surface antigens; LHBsAg: Human hepatitis B virus large surface antigens.
only just beginning to turn as mid-stage of larva 4 as 72 h post-hatching. Oogenesis was observed in some worms expressing SHBsAg until 96 h post-hatching (data not shown). Worms expressing MHbsAg and LHBsAg at 52 h and 68 h post-hatching showed a retardation of gonad development that was less severe than that observed in worms expressing SHBsAg at a similar stage (Figure 2, lower two rows). The severity of gonad retardation clearly reflected the reduced percentage of egg-laying (9%, 10%, and 54%, respectively) and average brood size (66, 175, and 239, respectively) in the three lines of transgenic worms, as shown in Table 1.

**DISCUSSION**

In this study, we demonstrated that transgenic worms expressing three forms of HBsAgs throughout the body exhibited lower rates of egg-laying, reduced brood sizes and retardation of gonad development to various degrees. Unexpectedly, worms expressing SHBsAg displayed the most severe defects (Table 1). No study has yet reported that the expression of SHBsAg can induce detectable phenotypes in cultured cells or animals; however, ER-stress and tumor formation have been observed in cells and animals expressing LHBSAg and MHbsAg[16,32]. Consistent with previous studies, worms expressing LHBSAg and MHBSAg were found to possess higher levels of enpl-1, hsp-4, pdi-3, and xbp-1 transcripts as did N2 worms pretreated with DTT (Figure 3). Because the gonad is the organ most sensitive to environmental changes, we suggest that ER-stress signal occurring autonomously or non-autonomously in the gonad can lead to gonad retardation, a reduced rate of egg-laying, and a smaller brood size in transgenic worms expressing LHBSAg and MHBSAg.

The unexpected results of the most severe pheno-
types induced by the expression of SHBsAg might be explained by the different nature of the three forms of HBsAg. In general, SHBsAg can form subviral particles of approximately 22 nm and be constantly secreted outside of cells whereas MHBsAg is less efficiently secreted and LHBsAg is usually retained in the ER \(^{[29,32]}\). This hypothesis is supported by the results shown in Figure 3, namely that four ER-stress related transcripts (enpl-1, hsp-4, pdi-3 and xbp-1) were substantially elevated in worms expressing LHBsAg and MHBsAg but only one transcript (pdi-3) displayed a slight elevation in worms expressing SHBsAg. The secretion of SHBsAg might either trigger signals inhibiting gonad development or titrate out secretion factors that are required for gonad development, although ER-stress signals might also play a minor role (Figure 4). Nevertheless, the underlying mechanism that leads to the most severe phenotypes in worms expressing SHBsAg remains unknown and will be elucidated by performing rescue and genetic cross experiments in the future.

* C. elegans has been used for studying viral pathogenesis and virus-host interaction for more than a
decade. In comparison with the number of publications using Caenorhabditis elegans to study viral, bacterial and fungal pathogenesis, relatively few papers have focused on viral pathogenesis and virus-host interaction in the past 10 years. The bottleneck could be due to the difficulty of creating transgenic worms expressing viral antigens. Currently, two methods for delivering ectopic genes into Caenorhabditis elegans are microinjection and gene bombardment, neither of which are easily achievable in general biology laboratories. To use Caenorhabditis elegans as a platform for studying virus-host interaction, virologists must collaborate with worm scientists. Alternatively, virologists could engineer the three viruses (Onsry, Santeu, and Le Blanc virus), that naturally infect Caenorhabditis elegans to become versatile vectors for the easy delivery of different viral genes into worms through infection.

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REFERENCES

1. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. Nature 1985; 317: 489-495 [PMID: 2995835 DOI: 10.1038/317489a0]
2. Ganem D, Varmus HE. The molecular biology of the hepatitis B virus. Annu Rev Biochem 1987; 56: 651-693 [PMID: 3039907 DOI: 10.1146/annurev.bi.56.070187.003251]
3. Locarnini S, Littlejohn M, Aziz MN, Yuen L. Possible origins and evolution of the hepatitis B virus (HBV). Semin Cancer Biol 2013; 23: 561-575 [PMID: 24013024 DOI: 10.1016/j.semcancer.2013.08.006]
4. Brechot C, Kremsdorf D, Sooussan P, Pimene P, Dejean A, Paterlini-Brechot P, Tiollais P. Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC): molecular mechanisms and novel paradigms. Pathol Biol (Paris) 2010; 58: 278-287 [PMID: 20667665 DOI: 10.1016/j.pathbio.2010.05.001]
5. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. Lancet 1981; 2: 1129-1133 [PMID: 6118576 DOI: 10.1016/S0140-6736(81)90585-7]
6. Wang J, Chenivesse X, Henglein B, Brechot C. Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. Nature 1990; 343: 555-557 [PMID: 1967822 DOI: 10.1038/34355a0]
7. Wang J, Zindy F, Chenivesse X, Lamas E, Henglein B, Brechot C. Modification of cyclin A expression by hepatitis B virus DNA integration in a hepatocellular carcinoma. Oncogene 1992; 7: 1653-1656 [PMID: 1321406]
8. Wang HC, Chang WT, Chang WW, Wu HC, Huang W, Lei HY, Lai MD, Fausto N, Su IJ. Hepatitis B virus pre-S2 mutant upregulates cyclin A expression and induces nodular proliferation of hepatocytes. Hepatology 2005; 41: 761-770 [PMID: 15726643 DOI: 10.1002/hep.20615]
9. Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Viral 2004; 78: 1275-12734 [PMID: 15542625 DOI: 10.1128/JVI.78.23.12725-12734.2004]
10. Yen TT, Yang A, Chiu WT, Li TN, Wang LH, Wu YH, Wang HC, Chen L, Wang WC, Huang W, Chang CW, Chang MD, Shen MR, Su IJ, Wang LH. Hepatitis B virus PreS2-mutant large surface antigen activates store-operated calcium entry and promotes chromosome instability. Oncotarget 2016; 7: 23346-23360 [PMID: 26992221 DOI: 10.18632/oncotarget.10299]
11. Tennant BC, Tosklov IA, Peek SE, Jacob JR, Menne S, Hornbuckle WE, Schinazi RD, Korba BE, Cote PJ, Gerin JL. Hepatocellular carcinoma in the woodchuck model of hepatitis B virus infection. Gastroenterology 2004; 127: S283-S293 [PMID: 15508906 DOI: 10.1053/j.gastro.2004.09.043]
12. Gerin JL, Cote PJ, Korba BE, Tennant BC. Hepadnavirus-induced liver cancer in woodchucks. Cancer Detect Prev 1989; 14: 227-229 [PMID: 2695243]
13. Trancy C, Fievez G, Robinson WS, Tiollais P, Marion PL, Buendia MA. Frequent amplification of c-myc in ground squirrel liver tumors associated with past or ongoing infection with a hepadnavirus. Proc Natl Acad Sci USA 1992; 89: 3874-3878 [PMID: 1570307 DOI: 10.1073/pnas.89.9.3874]
14. Hsu T, Mörty T, Ettemble J, Louise A, Tréco P, Tiollais P, Buendia MA. Activation of c-myc by woodchuck hepatitis virus insertion in hepatocellular carcinoma. Cell 1988; 55: 627-635 [PMID: 3180223 DOI: 10.1016/0092-8674(88)90221-8]
15. Wu BC, Li CC, Chen HJ, Chang JL, Jeng KS, Chou CK, Hsu MT, Tsai TF. Blocking of G1/S transition and cell death in the regenerating liver of Hepatitis B virus X protein transgenic mice. Biochem Biophys Res Commun 2006; 340: 916-928 [PMID: 16403455 DOI: 10.1016/j.bbrc.2005.12.089]
16. Chisari FV, Klopchin K, Moriyama T, Pasquinielli C, Dunsford HA, Sell S, Pinkert CA, Brinster RL, Palmeri RD. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell 1989; 59: 1145-1156 [PMID: 2598264 DOI: 10.1016/0092-8674(89)0770-8]
17. Kim A, Kwon OS, Kim SO, He L, Bae EY, Lee MS, Jeong SJ, Shim JH, Yoon DY, Kim CH, Moon A, Kim KE, Ahn JS, Kim BY. Caspase-3 activation as a key factor for HBx-transformed cell death.
Zhang XD, Wang Y, Ye LH. Hepatitis B virus X protein accelerates the development of hepatoma. *Cancer Biol Med* 2014; 11: 182-190 [PMID: 25364579]

Geng X, Harry BL, Zhou Q, Sken-Gaar RR, Ge X, Lee ES, Mitani S, Xue D. Hepatitis B virus X protein targets the Bel-2 protein CED-9 to induce intracellular Ca2+ increase and cell death in Caenorhabditis elegans. *Proc Natl Acad Sci USA* 2012; 109: 18465-18470 [PMID: 23091037 DOI: 10.1073/pnas.1204668109]

Brenner S. The genetics of Caenorhabditis elegans. *Genetics* 1974; 77: 71-94 [PMID: 4366676]

Ankeny RA. The natural history of Caenorhabditis elegans research. *Nat Rev Genet* 2001; 2: 474-479 [PMID: 11389464 DOI: 10.1038/sj/hmg/9603588]

Diogo J, Bratanich A. The nematode Caenorhabditis elegans as a model for study viruses. *Arch Virol* 2014; 159: 2843-2851 [PMID: 25009902 DOI: 10.1007/s00705-014-2168-2]

Kurz CL, Renshaw H, Frezal L, Jiang Y, Félix MA, Wang D. Engineering recombinant Orsay virus directly in the metazoan host Caenorhabditis elegans. *J Virol* 2014; 88: 11774-11781 [PMID: 25078701 DOI: 10.1128/JVI.01630-14]

Franz CJ, Renshaw H, Frezal L, Jiang Y, Félix MA, Wang D. Orsay, Santeuil and Le Blanc viruses primarily infect intestinal cells of Caenorhabditis nematodes. *Front Microbiol* 2014; 5: 348 [PMID: 25216563 DOI: 10.3389/fmicb.2014.00348]

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