PNAS RESEARCH ARTICLE | PHYSIOLOGY

Gene delivery available in molluscan cells by strong promoter discovered from bivalve-infectious virus

Jeongwoong Yoon[a], Wen-Bin Gu[a], Mizuki Konuma[a], Mutsuko Kobayashi[a], Hayato Yokoi[b], Makoto Osada[a], and Kazue Nagasawa[a,1]

Edited by Stephen Goff, Columbia University Irving Medical Center, New York, NY; received June 13, 2022; accepted September 13, 2022

Understanding gene functions in marine invertebrates has been limited, largely due to the lack of suitable assay systems. Such a system requires investigative methods that are reproducible and can be quantitatively evaluated, such as a cell line, and a strong promoter that can drive high expression of a transgene. In this study, we established primary cell culture from a marine bivalve mollusc, *Mizuhopecten yessoensis*. Using scallop primary cells, we optimized electroporation conditions for transfection and carried out a luciferase-based promoter activity assay to identify strong promoter sequences that can drive expression of a gene of interest. We evaluated potential promoter sequences from genes of endogenous and exogenous origin and discovered a strong viral promoter derived from a bivalve-infectious virus, ostreid herpesvirus-1 (OsHV-1). This promoter, we termed OsHV-1 promoter, showed 24.7-fold and 16.1-fold higher activity than the cytomegalovirus immediate early (CMV IE) promoter and the endogenous EF1α promoter, the two most commonly used promoters in bivalves so far. Our GFP assays showed that the OsHV-1 promoter is active not only in scallop cells but also in HEK293 cells and zebrafish embryos. The OsHV-1 promoter practically enables functional analysis of marine molluscan genes, which can contribute to unveiling gene-regulatory networks underlying astonishing regeneration, adaptation, reproduction, and aging in marine invertebrates.

marine invertebrates | CMV promoter | OsHV-1 | electroporation | primary culture

Significance

Gene delivery is a fundamental tool to study functions and associated factors of a gene of interest. A key element in successful gene delivery is a strong promoter sequence, which drives its downstream gene expression by interacting with cellular transcription factors. In marine molluscs, strong promoters are lacking. Strong promoters are often discovered from immediately early genes of infectious viruses. We evaluated the activities of putative promoter sequences from bivalve-infectious virus OsHV-1 genes, using Yesso scallop primary cells we established. As a result, we found a highly active promoter in molluscan cells. This viral promoter showed 25-fold higher activity than the cytomegalovirus immediate early (CMV IE) promoter, the most commonly used promoter in bivalves so far.

Model organisms from terrestrial and freshwater species have expanded our knowledge about functions and evolution of genes. In stark contrast, understanding gene functions of marine invertebrates has been limited, likely due to the difficulty with access to them and incompatibility of conventional experiment methods (1). Nonetheless, their distinct properties, such as high regenerative capacity (2), osmotic and thermal tolerance/adaptivity (3), sexual plasticity (4), and extreme lifespan (5), make them attractive models for a deeper understanding of comparative physiology and evolutionary genetics. Among marine invertebrate phyla, molluscs are one of the most-successful animal groups during evolution in terms of species diversity (6). In this study, we focused on bivalve molluscs, the second-largest class in the phylum Mollusca and in many cases of commercial importance as food (6). With regard to bivalve gene functions, functional analysis by deliberate modifications of gene expression has been barely carried out. To fill the knowledge gap between model organisms and marine molluscs, we reason that the primary requirements are (i) investigative methods that are reproducible and can be quantitatively evaluated, such as a cell line, and (ii) a strong promoter sequence that can drive high expression of a transgene.

cis-regulatory elements are short DNA sequence motifs flanking coding regions (also known as coding DNA sequence [CDS]) of genes. They regulate gene transcription of neighboring gene(s) by binding to transcription factors (7). Among cis-regulatory elements, promoters are DNA sequences required for the initiation of transcription of neighboring gene(s), and in genetic research, promoters are fundamental research tools to decipher gene functions. The efforts from the 1990s to transfect bivalve cells have not been very successful because of the low efficiency of transfection methods and the lack of strong promoters (8). In bivalve cells and embryos, the promoters tested so far are 5′ upstream regions of *Drosophila hsp70* (9, 10), *Crassostrea gigas actβ* (11), *ef1α* (12–14), the long terminal repeats of Molony murine leukemia virus (9, 15), and the cytomegalovirus immediate early (CMV IE) promoter (14, 16, 17), but practically reliable promoters have yet to be discovered. Recently, applications of genome-editing techniques are advancing in bivalve research (13, 18). However, with the absence of efficient promoters, those studies are compelled to rely on direct delivery of genetic material by microinjection into small bivalve eggs (18) or electroporation with a conventional promoter (13).

Strong promoters are often discovered from viral genes, as exemplified in the widely used CMV IE (mammals), opIE2 (insects), and CaMV 35S (plants) promoters (19–21).

Author affiliations: [a]Laboratory of Aquaculture Biology, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-8572, Japan; and [b]Laboratory of Fish Genetics, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-8572, Japan

Author contributions: J.Y., M.O., and K.N. designed research; J.Y., W.-B.G., M. Konuma, M. Kobayashi, and H.Y. performed research; J.Y. and K.N. analyzed data; and J.Y., H.Y., and K.N. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: kazue.nagasawa.d6@tohoku.ac.jp.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2209910119/-/DCSupplemental.

Published November 2, 2022.

Viruses depend on the cell machinery of host cells to replicate themselves. In particular, viral immediate early genes, the first genes transcribed in the absence of other viral gene products, can possess promoter sequences that can be easily recognized and transcribed by the host cell (22). Recently, the *immediate early 1* (*ie1*) promoter of white spot syndrome virus (WSSV), a notorious pathogen causing severe mortalities in aquaculture shrimps, was shown to achieve efficient transfection in shrimp cells (23, 24). Inspired by the above examples, we considered a possibility that promoters of ostreid herpesvirus-1 (OsHV-1), a virus affecting different bivalve species, particularly the Pacific oyster *C. gigas* (25), could be a breakthrough in gene delivery into molluscan cells.

Cultivatable bivalve cells are desired for various research purposes. For example, they are desired for studying host–pathogen interactions (26), pearl formation (27), biomonitoring (28), and, more recently, bivalve transmissible neoplasia (29). For our purpose, cell culture is necessary for the evaluation of different promoter activities. Unfortunately, despite decades of efforts, no cell line has been established from any marine invertebrates, including marine molluscs (30, 31). Attempts to date (*SI Appendix*, Table S1) suggest that the difficulties in culturing marine molluscan cells lie in facing frequent contamination of marine-derived micro-organisms, obtaining proliferating cell populations, and finding adequate physicochemical and physiological conditions for molluscan cells.

The Yesso scallop, *Mizuhopecten yessoensis* (Jay, 1857), is a subtidal species inhabiting cold seabed of the Northwest Pacific. Besides being commercially important, *M. yessoensis* is a desirable model to study marine molluscan reproduction in that it has a massive, discrete, and exposed gonad with gonochoristic characteristics (32), and it is one of the few bivalve molluscs whose complete genome sequence is available (33). In this study, we aimed to establish an in vitro transfection system for marine molluscan cells using electroporation and *M. yessoensis* cells. To achieve it, we (i) established primary cell culture, (ii) optimized electroporation conditions, and (iii) carried out reporter gene assays to find a strong promoter. As a result, we present a strong viral promoter derived from an OsHV-1 gene, which is highly active not only in molluscan cells but also in mammalian and fish cells. This promoter, we termed OsHV-1 promoter, makes gene delivery and functional analysis of a gene of interest practically available in marine molluscan cells.

Results

Primary Culture Was Established for Scallop Heart Cells and Hemocytes. To establish a scallop cell culture system, we examined several tissues from *M. yessoensis* as listed in *SI Appendix*, Fig. S1. In brief, the most successful cell cultures were the heart explant and the immature gonad explant culture, in which fibroblast-like cells vigorously migrated out of the tissue fragments and remained healthy for about a month. Other scallop cells, including hemocytes, were not maintained in vitro as long, due to the rapid degeneration and severe contamination with small protozoans, which were not addressed by antibiotics (*SI Appendix*, Fig. S1).

We established primary culture of scallop heart cells and hemocytes for reporter gene assays. For heart cells, from a single scallop, ~100 mg (wet weight) of heart (ventricle) was collected. In the first attempt of cell preparation, with enzymatic dissociation alone, cells poorly attached to the substrate and started degenerating within a week. In the next attempt, explant culture without trypsinization also failed to attach or yield migrating cells. The breakthrough was made by using trypsinized explants, by which cells vigorously migrated from the seeded tissue fragments, leading to successful primary culture (Fig. 1 *A*–*F* and *SI Appendix*, Fig. S2). In culture, heart-cell populations were heterogenous and various in size, in which a majority of cells were fibroblast-like or macrophage-like (Fig. 1*E*), and beating explants were occasionally observed (*SI Appendix*, Movie S1). Scallop primary heart cells were in general viable up to 3 to 4 wk at 20 °C and tended to degenerate gradually afterward, but some remained quiescent but viable for more than 3 mo (Fig. 1*G*).

For hemocytes, invertebrate blood cells, about 2 mL of hemolymph was withdrawn from a single scallop with the cell density ~4×10^6 hemocytes/mL (Fig. 1*H*). Hemocytes readily attached to the substrate within hours after seeding, and homogenous populations of highly mobile cells were observed (Fig. 1*I*). The presence of hemocytes is unlikely in the heart cell culture, considering the facts that minced heart fragments were thoroughly washed with artificial seawater, dodecyl benzene sodium sulfonate (DBSS), and trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Table 1) and that the observed cell size in the heart explant culture was much larger than hemocytes (Fig. 1 *E* and *I*). Primary hemocytes were viable up to 2 wk at 20 °C, and their degeneration was accelerated by medium replacement. In cultures of heart cells and hemocytes, additional supply of CO_2 or humidity was unnecessary.

For scallop cell culture conditions, Leibovitz's L-15 medium (L-15) was used as the basal medium based on previous studies (*SI Appendix*, Table S1). With L-15, we evaluated how seeding density, incubation temperature, osmolality, and fetal bovine serum (FBS) supplementation affected scallop cells in culture (*SI Appendix*, Fig. S2). Among 24 comparisons in total, some of the comparisons exhibited significant difference. In terms of cell elongation, the media with 10% FBS (900, 1,100 mOsmol/kg) were better than the medium without FBS, the medium of 1,100 mOsmol/kg was better than that of 900 mOsmol/kg, and the seeding density 4×10^5 cells/mL was better than 2×10^5 cells/mL (*SI Appendix*, Fig. S2). Although FBS seemed to help scallop cell maintenance, supplementation of 2% FBS was sufficient and higher concentrations were not more beneficial, at least for heart cells. The culture conditions determined based on these data were used throughout the study.

The risk of microbial contamination was our primary concern in scallop cell culture. Any culture attempts without the use of antibiotics failed within a week, due to bacterial and fungal contamination. Direct supplementation with 200 U/mL penicillin and 200 μg/mL streptomycin, or 25 μg/mL rifampicin alone, in the growth medium effectively addressed bacterial contamination. We found that direct inclusion of amphotericin B, an antifungal, in the growth medium was detrimental to scallop primary cells (*SI Appendix*, Fig. S2), which was different from what has been routinely done (*SI Appendix*, Table S1). Instead, inclusion of amphotericin B in the disinfection solution (Table 1) did not affect cell viability or attachment yet effectively addressed fungal contamination. At the initiation of cell culture, contamination with some small, suspending protozoans was inevitable in all scallop cell cultures tested. Those microalgae-like protozoans did not appear to harm primary cells and were gradually removed by medium replacement.

Electroporation Conditions Were Optimized for Scallop Cells. For the reporter gene assays, electroporation was chosen as the method for transfection, given the bromodeoxyuridine (BrdU) immunoassay results showing that there are few mitotic heart

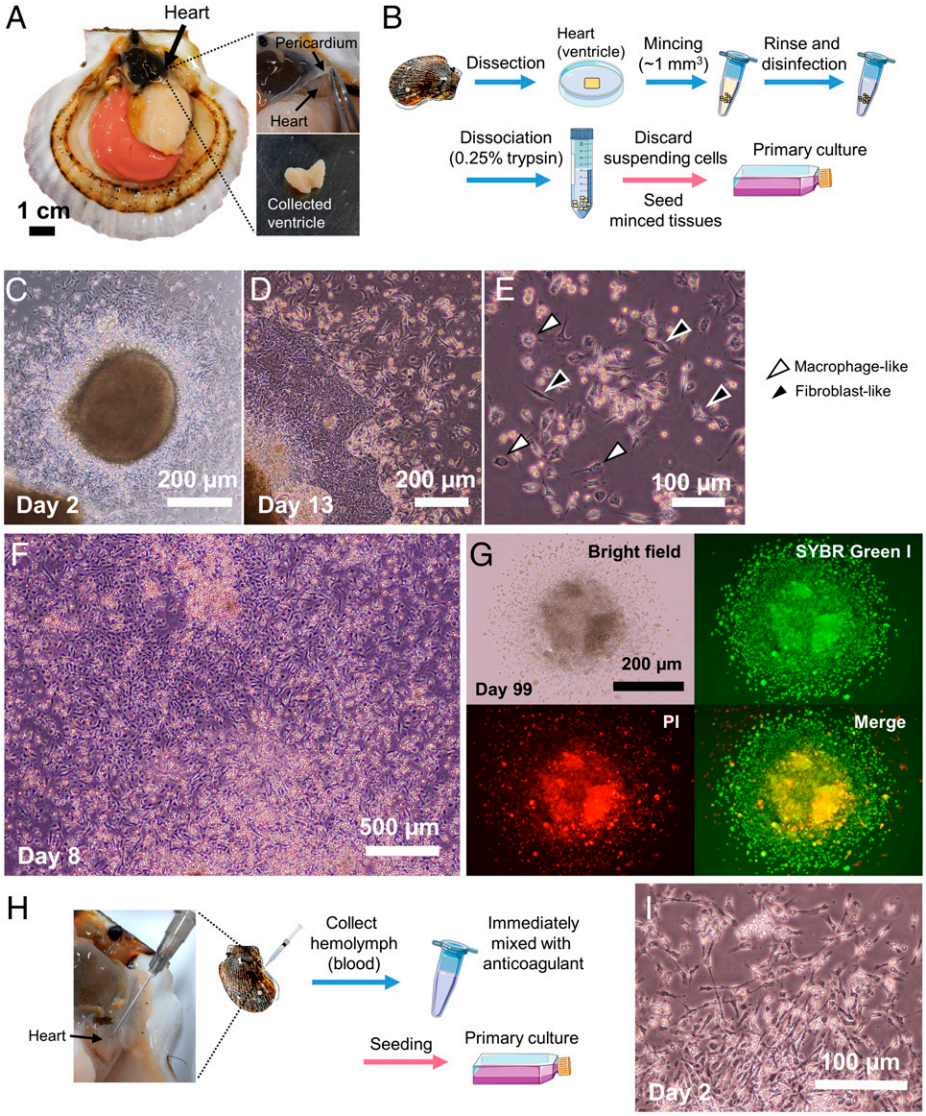

Fig. 1. Primary culture for scallop heart cells and hemocytes. (*A*) Soft body of the Yesso scallop, *Mizuhopecten yessoensis*. The left shell, left mantle, and gill were removed. The heart is located in the dorsal posterior, protected by the pericardium (*Inset*). The ventricle, the bright part of the heart, was collected for cell culture. (*B*) A schematic of scallop heart cell preparation. (*C*–*F*) Scallop heart cells in culture. (*C* and *D*) Scallop heart cells on day 2 (*C*) and day 13 (*D*). (*E*) A magnified view of *D*, to highlight cell morphology. A majority of primary heart cells showed fibroblast-like or macrophage-like morphology. Cells of similar morphology were often different in size. (*F*) The primary heart cells formed a monolayer on day 8. (*G*) Scallop heart cells on day 99, stained with SYBR I for live cells and propidium iodide (PI) for dead cells. On some occasions, cells are quiescent yet still alive for more than 3 mo. (*H*) A schematic of scallop hemocyte preparation. Hemolymph (blood) was collected directly from the heart. (*I*) Hemocytes in culture on day 2.

cells (*SI Appendix*, Fig. S3). While a few studies carried out electroporation on bivalve embryos (13, 14, 16), there is no report of electroporation on molluscan primary cells. Thus, we first tested whether scallop primary cells could be transfected by electroporation using luciferase-encoding mRNA and optimized basal electroporation conditions (Fig. 2*A*).

In preliminary experiments, we examined how different conditions in poring pulse, duration, and buffer osmolality affected cell viability using dissociated scallop heart cells. With original Opti-MEM, approximately of 300 mOsmol/kg, cell viability was poor even with low electrical stress (Fig. 2*B*). Cell viability was improved by 36.4% on average after the adjustment of osmolality to ∼1,050 mOsmol/kg (Fig. 2*B*).

Scallop heart cells were electroporated with luciferase messenger RNA (mRNA) with suitable electroporation conditions suggested by the cell viability data. At 3 d postelectroporation (dpe), the morphology and number of cells in culture were not noticeably different among the treatments (Fig. 2*C*). The luciferase assay results show that scallop heart cells were successfully transfected with luciferase-encoding mRNA by electroporation (Fig. 2*D*).

OsHV-1-Derived Poshv088 and Poshv117 Are Highly Functional Promoters in Scallop Cells. To identify a strong promoter in molluscan cells, we examined 11 putative promoter regions from endogenous genes of *M. yessoensis* and genes of a bivalve-infectious virus, OsHV-1, and a shrimp-infectious virus, WSSV (Fig. 3*A*). OsHV-1-infected oysters have a high copy number of OsHV-1 transcripts (34), from which it is implied that some OsHV-1 genes have a strong promoter sequence highly functional in molluscan cells. From the total 136 annotated OsHV-1 open reading frames (ORFs) (25), we selected six OsHV-1 genes *oshv027*, *oshv029*, *oshv072*, *oshv080*, *oshv088*, and *oshv117* as candidate genes that possess a strong promoter sequence, based on the temporal postinfection OsHV-1 gene expression data from Segarra et al. (35) and the summary of

Table 1. Formulation of solutions and growth medium used in the present study

Solution*	Formulation	References
Molluscan balanced salt solution (MBSS)	NaCl 26.22 mg/mL KCl 1.08 mg/mL $MgCl_2$-$6H_2O$ 4.7 mg/mL $MgSO_4$-$7H_2O$ 6.52 mg/mL $NaHCO_3$ 0.3 mg/mL NaH_2PO_4-$2H_2O$ 0.058 mg/mL $CaCl_2$-$2H_2O$ 1.48 mg/mL Glucose 0.3 mg/mL	(27)
Molluscan Ca^{2+}/Mg^{2+}-free saline (MCMFS)	NaCl 26.22 mg/mL KCl 1.08 mg/mL $NaHCO_3$ 0.3 mg/mL NaH_2PO_4-$2H_2O$ 0.058 mg/mL Glucose 0.3 mg/mL	
Trypsin-EDTA solution	Trypsin 0.25% (Gibco) EDTA-2Na 1.15 mg/mL in MCMFS	
Disinfection BSS (DBSS)	Penicillin-Streptomycin 400 U/mL (Gibco) Amphotericin B 2.5 μg/mL (Sigma-Aldrich) in MBSS	
Growth medium	Leibovitz's L-15 medium (Gibco) Fetal bovine serum (FBS) 2% (Gibco) Penicillin-Streptomycin 200 U/mL (Gibco) in 75% MBSS	
Modified Alsever's solution (MAS)	NaCl 22.50 mg/mL Trisodium citrate-$2H_2O$ 8.0 mg/mL Glucose 20.80 mg/mL EDTA-2Na 3.36 mg/mL	

MBSS, molluscan balanced salt solution; MCMFS, molluscan calcium/magnesium-free saline; DBSS, disinfection BSS; MAS, modified Alsever's solution.
*All balanced salt solutions and growth medium were adjusted to pH 7.4 with HCl and NaOH.

oyster RNA sequencing data from Rosani and Venier (34) (*SI Appendix*, Fig. S4).

To evaluate the promoter activities in primary cells with a luciferase assay, we first assessed the correlation between relative luminescence unit (RLU) of luciferase and cell counts with Pmy-ef1α, Poshv088, and Poshv117 (Fig. 3 *B* and *C*). With Poshv088 and Poshv117, highly active promoters, R^2 values were 0.95 and 0.54, suggesting that cell number and luciferase expression were positively correlated. Although Pmy-ef1α, a weak promoter, had R^2 value of 0.3 (Fig. 3*C*), we concluded that direct cell counts were suitable for normalization of luciferase expression.

In our promoter activity assay, Poshv117 showed the highest activity in scallop heart cells, followed by Poshv088 (Fig. 3*D*). The CMV IE promoter and Pmy-ef1α showed some activity but significantly lower than Poshv117 or Poshv088. The expression level of Poshv117 was 16.1- and 24.7-fold higher than Pmy-ef1α and the CMV IE promoter (Fig. 3*D*). Other promoters, including Pwsv-ie1 and Pwsv465, showed negligible promoter activities in scallop heart cells.

Using Poshv117, we further optimized electroporation conditions for scallop heart cells (*SI Appendix*, Fig. S5). When electroporated with varying voltage, the 200 V–treated group showed higher RLU than those with lower voltage (Fig. 3*E*). We also found that transgene expression significantly changed over 6 d after electroporation. The peak was observed at 2 dpe, and RLU decreased gradually afterward (Fig. 3*F*). Different impedance did not affect RLU, and there were more viable cells in lower impedance groups (38 and 42 Ω) as compared with 60 Ω (*SI Appendix*, Fig. S5). In addition, luciferase expression increased in a dose-dependent manner with plasmid DNA concentration in the buffer (*SI Appendix*, Fig. S5).

Poshv117 Is Active in Molluscan, Fish, and Mammalian Cells. Poshv088 and Poshv117 showed the highest activities from our promoter assay among the tested 13 expression vectors (Fig. 3*D*). We further tested whether Poshv088 and Poshv117 could actually induce strong GFP expression in molluscan and nonmolluscan cells, using scallop heart cells, scallop hemocytes, human HEK293 cells, and zebrafish embryos.

To represent scallop cells, primary heart cells and hemocytes were used for GFP assays. As hemocytes could not be electroporated using the cuvette-based electroporation system due to high mortality after electroporation, we employed lipofection to transfect attached hemocytes.

Poshv117 was highly active in scallop cells and also active in HEK293 cells and zebrafish embryos, whereas Poshv088 failed to induce strong GFP signal in any of them. Scallop heart cells showed visible GFP expression at 2 dpe (Fig. 4*B*). Because scallop heart cells emit high autofluorescence (Fig. 3*B*), by immunofluorescence with anti-GFP antibody, we verified that the fluorescence from electroporated scallop cells was true GFP signal (Fig. 4*B*). Scallop hemocytes showed GFP expression from 1 d postlipofection (dpl) (Fig. 4*C*). For HEK293 cells, GFP-positive cells were observed at 1 dpe (Fig. 4*D*), and there was no noticeable change in GFP intensity or cell viability in culture for a week. Microinjected zebrafish embryos showed granular GFP expression at 24 h postinjection (hpi) (Fig. 4*E*). From the validated functionality of Poshv117 in molluscan and nonmolluscan cells, we termed this promoter OsHV-1 promoter.

OsHV-1 Promoter (Poshv117) Possesses Conserved Eukaryotic Transcription Motifs. The DNA sequences of the OsHV-1 promoter and the *oshv117* CDS were analyzed in silico. Fig. 5*A* shows the positions of *oshv117* and its neighboring genes. In the OsHV-1 reference genome (25), there are 4,600-bp identical duplicates in which *oshv116*, *oshv117*, *oshv118*, and *oshv119* are located. The duplicated regions are 100% identical in DNA sequence and exist in a complementary fashion (i.e., one in 5′–3′ and the other in 3′–5′ direction).

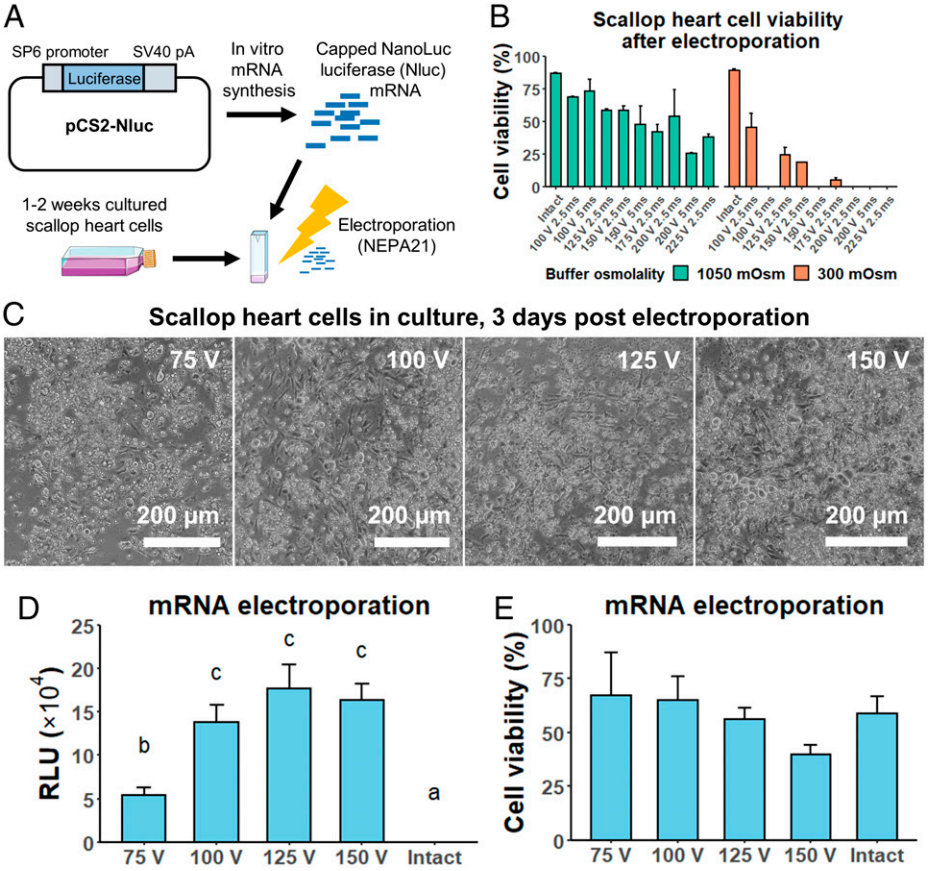

Fig. 2. Optimization of electroporation conditions with luciferase mRNA. (*A*) A schematic of scallop cell electroporation with luciferase-encoding mRNA. The electroporation conditions were optimized by transfection with luciferase mRNA. (*B*) Cell viability of 1×10^6 scallop heart cells after electroporation without mRNA, with different pulse voltage (V), pulse length (ms), and buffer osmolality (mOsmol/kg) ($n = 2$). (*C–E*) Luciferase mRNA electroporation results. The poring duration was fixed to 2.5 ms in all treatments. (*C*) Electroporated heart cells on day 3. The morphology and number of cells in culture were not noticeably different among the treatments. (*D*) Luciferase expression in scallop heart cells electroporated with mRNA at 3 d postelectroporation ($n = 3$). (*E*) Cell viability immediately after electroporation ($n = 2$). Different letters (a–c) indicate statistically significant difference ($P < 0.05$).

The OsHV-1 promoter is comprised of 5′ untranslated region (UTR) of *oshv117* (233 bp), full CDS (669 bp), and 5′ UTR of *oshv118* (167 bp). Considering a possibility that CDS of a gene could be a cis-regulatory element for a neighboring gene (36), we included the CDS of *oshv118* for the sequence analysis as a potential reservoir of cis-regulatory elements for *oshv117*.

The sequence analysis of the OsHV-1 promoter by Neural Network Promoter Prediction (NNPP) and NSite is presented in Fig. 5*B*. Transcription initiation sites were predicted by NNPP, a neural network–based promoter prediction program, at the positions −58 and −30 upstream from the start codons of *oshv117* and *oshv118*. For *oshv117*, TATA box (TATATAA) was found −30 upstream from the transcription initiation site. NSite is a consensus-based search tool for putative functional motifs. The following is the binding motifs proposed by NSite within the OsHV-1 promoter for known regulatory elements, in which lower letters indicate mismatches with the consensus sequence: TACGTGGG, a conserved sequence that binds to mammalian hypoxia-inducible factor 1 (HIF-1) (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*) (37); GGATTGGC, a putative binding site of mammalian dihydrofolate reductase (DHFR) (*M. musculus*) (38); GAGGGAagGT, a putative binding site of mammalian mammary cell–activating factor (MAF) (*H. sapiens* and *M. musculus*) (39); and ACACCatTACATT, near the TATA box for *oshv117*, a RPG box-like sequence that putatively binds to Ras-associated protein 1 (RAP1) of *Saccharomyces cerevisiae* (40). The CAATT box consensus sequence (T/C)GATTGG(T/C)(T/C)(G/A) (41) was found at the position −91 5′ upstream from the *oshv117* transcription initiation site. The Kozak consensus sequence (A/G)NNATG(A/G) was conserved around the start codon of *oshv117* (42). Also, indirect (TTCCCTGGT and GCCAGGGAA, AATGCGT and ACGCATT) and direct palindromic sequences (AACATGTT, AAATATTT, TCCATATGGA, TGATATCA) and three directly repeated sequences (CAA CAACAA, CTGTATCTGTAT, AACAACAACAAC) were found. The palindromic sequences were no longer than 10 bp separately and 18 bp in combination.

We investigated which part of the OsHV-1 promoter grants its promoter activity in molluscan cells. When luciferase expression was driven by (i) full CDS of *oshv118* (669 bp) + full 5′ UTR of oshv117 (233 bp), (ii) partial CDS of *oshv118* (354 bp) + full 5′ UTR of oshv117 (233 bp), (iii) full 5′ UTR of oshv117 (233 bp), and (iv) partial 5′ UTR of oshv117 (151 bp), RLU was 61.1%, 30.3%, 11.2%, and 0.2% respectively, as compared with the full OsHV-1 promoter sequence (1,069 bp) (Fig. 5*C*).

For the protein sequence, the *oshv117* CDS contained a RING finger (RING1) domain with the consensus motif Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys (43) (Fig. 5*D*). There were no other noticeable motifs in the *oshv117* CDS.

Discussion

In this study, our objective was to establish a system that allows efficient gene delivery into molluscan cells. To achieve it, we

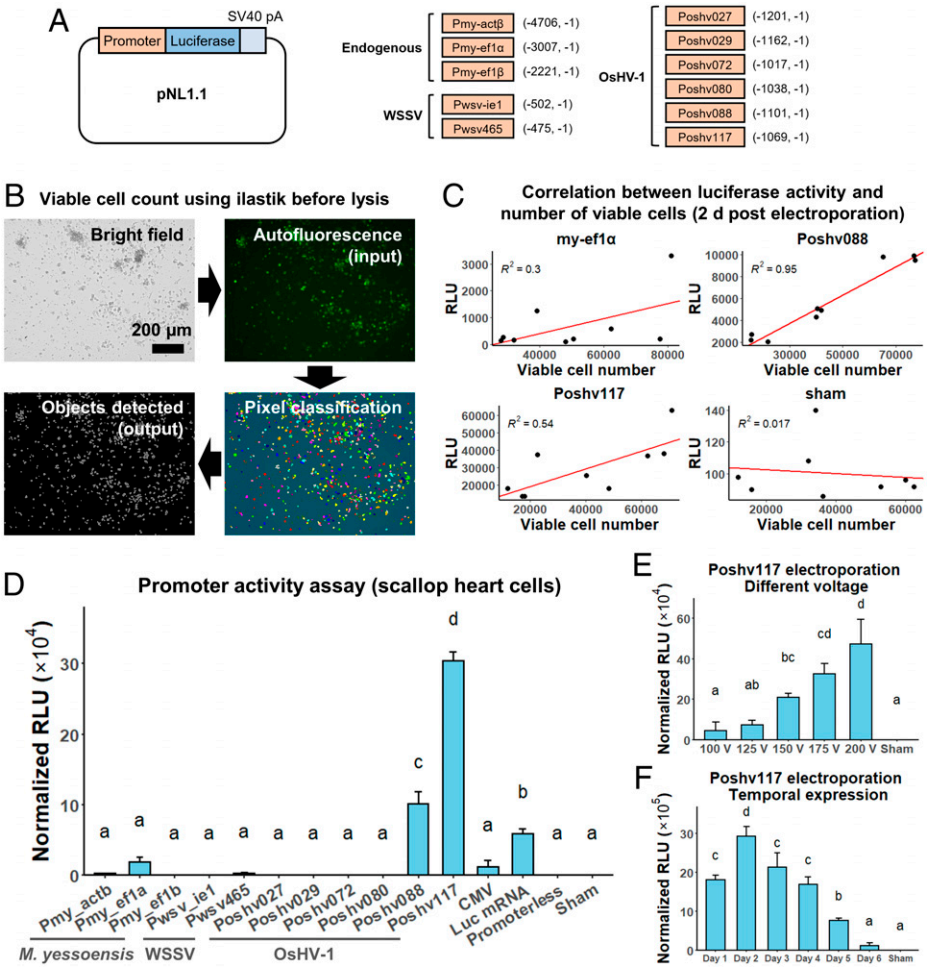

Fig. 3. Promoter activity assay by electroporation with plasmid DNA. (*A*) DNA constructs in which luciferase expression is driven by different promoters. Promoters of Yesso scallop endogenous (Pmy-actβ, Pmy-ef1α, and Pmy-ef1β) and viral exogenous (WSSV-derived Pwsv-ie1, Pwsv465; OsHV-1-derived Poshv027, Poshv029, Poshv072, Poshv080, Poshv088, and Poshv117) genes were tested. (*B*) Autofluorescence of scallop primary heart cells and cell counting by ilastik. Autofluorescence emitted from scallop heart cells was photographed with long exposure time and run through a supervised learning pipeline for cell counting. (*C*) Linear correlation between luciferase expression and the number of adherent cells at 2 d postelectroporation. (*D*) Promoter activities of 11 DNA constructs plus pNL1.1 (promoterless), pNL1.1-CMV, and luciferase mRNA at 2 d postelectroporation were quantified by a luciferase assay ($n = 3$). (*E* and *F*) The Poshv117-luciferase vector was used for further optimization of electroporation. (*E*) Electroporation with different voltage ($n = 3$). (*F*) Temporal change of luciferase expression over 6 d postelectroporation ($n = 3$). Different letters (a–d) indicate statistically significant difference ($P < 0.05$).

sequentially (i) established primary cell culture, (ii) established electroporation conditions, and (iii) discovered a strong viral promoter and succeeded in inducing strong reporter gene expression.

We established primary culture of *M. yessoensis* heart cells and hemocytes after testing different types of cells. In culturing scallop primary cells, there were two barriers to overcome.

First, the risk of microbial contamination was the main difficulty. Being filter feeders, bivalves tend to bioaccumulate various contaminants in their tissues, namely marine bacteria, fungi, and protozoans. In our cell culture, the contamination issue was only partly addressed by the use of a combination of antibacterial and antifungal agents and by targeting a relatively clean organ like the heart. In fact, facing severe microbial contamination is not uncommon in primary cell culture of filter-feeding marine invertebrates, including sponges (44, 45), tunicates (46), and bivalve molluscs (47).

Second, *M. yessoensis* is a permanently subtidal species, and the information for cell culture from subtidal bivalve species was limited. To date, cell-culture attempts mainly focused on freshwater or intertidal bivalve species represented by oysters (e.g., *Crassostrea* spp.) and mussels (e.g., *Mytilus* spp.), accounting for 25 of 29 studies listed in *SI Appendix*, Table S1. Intertidal species are tolerant to a broad range of osmotic pressures, as shown in the successful culture of *Crassostrea virginica* cells at 425 mOsmol/kg (48). Studies on other marine invertebrates suggest that marine invertebrate cells can deal with a range of osmolality (49, 50) or even lower osmolality than that of their internal fluid (51, 52). We show that scallop cells can be cultured at 900–1,100 mOsmol/kg, approximately the osmolality of seawater. Aside from osmolality, we applied cell-culture conditions for intertidal bivalves to our study (*SI Appendix*, Table S1), namely L-15 medium, incubation at 20 °C, cold trypsinization for dissociation, and supplementation with FBS. These conditions worked reasonably well, if not optimal, as the same growth medium was able to maintain several different scallop cells (*SI Appendix*, Fig. S1).

In successful culture flasks, scallop heart cells formed a monolayer within 1 to 2 wk. It was difficult to assess cell growth quantitatively because cultured heart cells only resulted from explant culture, and there were high variations in cell conditions among the explants and culture flasks. That is, neither every seeded explant attached to the substrate nor the number and types of cells were always the same. Although the number of cells increased in culture, our BrdU assay results suggest that few scallop heart cells are mitotic, in agreement with previous

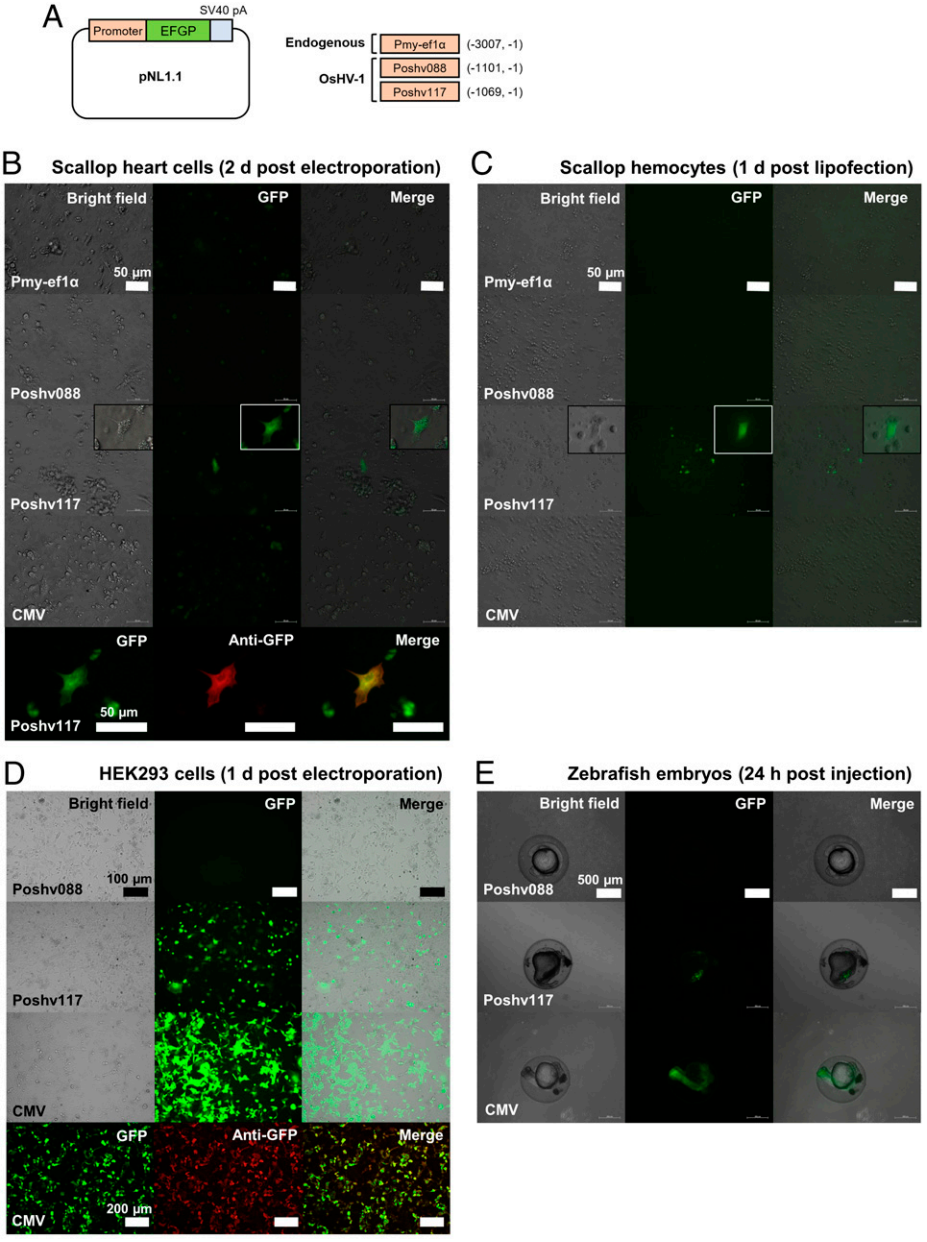

Fig. 4. OsHV-1-derived promoter (Poshv088 and Poshv117) activities in scallop cells, mammalian cells, and fish embryos. (*A*) DNA constructs in which EGFP expression is driven by Pmy-ef1α, Poshv088, or Poshv117. The four best promoters from the promoter activity assay, Pmy-ef1α, Poshv088, Poshv117, and the CMV IE promoter were further tested by GFP assays. (*B*) Scallop heart cells at 2 d postelectroporation with EGFP vectors driven by Pmy-ef1α, Poshv088, Poshv117, or the CMV IE promoter. *Insets* highlight the morphology of transfected heart cells. Among them, only Poshv117 drove strong GFP expression in scallop heart cells. The GFP signal was verified by immunofluorescence with anti-GFP antibody. In the merged photograph of GFP and RFP, green signals shown are autofluorescence by heart cells. (*C*) Scallop hemocytes at 2 d postlipofection with the same EGFP vectors. *Insets* highlight the morphology of transfected hemocytes. (*D* and *E*) HEK293 cells (*D*) at 1 d postelectroporation and Zebrafish embryos (*E*) at 24 h postinjection with EGFP vectors driven by Poshv088, Poshv117, or the CMV IE promoter. The CMV IE promoter was used as a positive control. Poshv117 drove GFP expression in HEK293 cells and zebrafish embryos.

studies on *Mya arenaria* and *Ruditapes decussatus* heart cells (53, 54). Thus, cultured heart cells were unlikely proliferating but simply migrating.

We here present successful transfection of scallop primary cells by electroporation with mRNA and plasmid DNA. We employed electroporation for transfection, a highly effective transfection method for a variety of cells (55), considering the low proliferation rate, i.e., a less chance of the nuclear envelope breakdown, of scallop cells. In molluscs, electroporation has been used only in oyster embryos (13, 16), and we report that molluscan primary cells were successfully electroporated with reporter genes.

After we confirmed that it was possible to electroporate scallop cells with mRNA, we proceeded with electroporation with plasmid DNA to search for a strong promoter. Because many functional viral promoters are approximately of 500- to 700-bp length (19–21, 56), we cloned roughly 1,000-bp 5′-upstream regions of OsHV-1 genes to include the full viral promoter sequences. In addition to six OsHV-1 genes, 5′-upstream regions of scallop endogenous *my-actβ*, *my-ef1α*, and *my-ef1β* and WSSV *wsv-ie1* and *wsv465* sequences were tested as potential promoters. *my-ef1α* and *my-actβ* have constitutive expression in scallop cells and are commonly used as a reference gene for normalization of gene expression (57). *my-ef1β* has high and stable expression in different life stages and was recently reported as a suitable reference gene (57). Pwsv-ie1 is known to be functional not only in shrimp and insect cells (24) but also

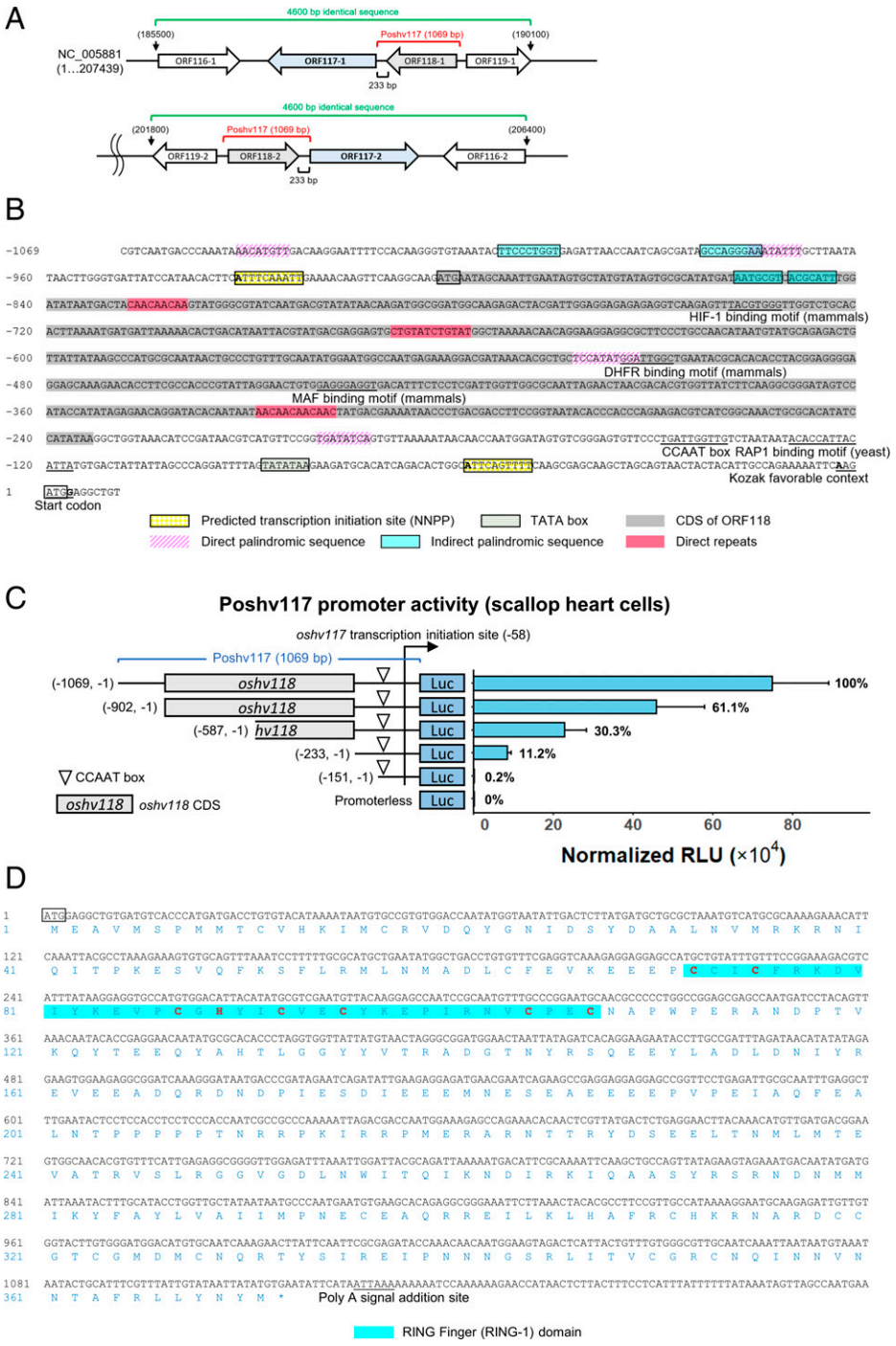

Fig. 5. Gene location and motif analysis of the OsHV-1 promoter (Poshv117) and *oshv117* coding region. (*A*) Position of *oshv117* (ORF117-1 and ORF117-2) in the OsHV-1 genome, in which there are 4,600 bp duplicated regions of 100% identical DNA sequence. OsHV-1 genes *oshv116*, *oshv117*, *oshv118*, and *oshv119* are located in the duplicated regions, which exist in a complementary fashion. (*B*) Organization and location of predicted regulatory motifs in the DNA sequence of the OsHV-1 promoter (Poshv117), −1,069 5′ upstream region of *oshv117*. The OsHV-1 promoter is composed of 5′ UTR of *oshv117* (233 bp), full coding region (669 bp), and partly 5′ UTR of *oshv118* (167 bp). The CCAAT box and TATA box are located −91 and −30 5′ upstream from the putative oshv117 transcription initiation site. In parentheses, the studied species of the putative binding factors are noted. "Yeast" refers to *Saccharomyces cerevisiae*, and "mammals" refers to *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, and/or *Cricetulus griseus*. (*C*) Promoter activities of the OsHV-1 promoter subregions, which are combinations of full, partial, or no *oshv118* coding region and full or partial *oshv117* 5′ UTR. (*D*) Coding region of *oshv117*. A conserved RING finger domain is located.

in fish (58) and mammalian cells (59). Considering its high activity in different groups of animals, Pwsv-ie1 was included in the assay, together with Pwsv465, of which promoter activity was higher than Pwsv-ie1 in Sf9 cells (56).

As a result of screening, we discovered a strong viral promoter from (−1,069 and −1) 5′-upstream region from the *oshv117* translation initiation site. This promoter, we termed OsHV-1 promoter, was functional not only in scallop cells but also in HEK293 cells and zebrafish embryos. At present, no report exists for OsHV-1 infection in vertebrates, despite the worldwide infection of OsHV-1 in aquaculture bivalve species (60, 61).

The OsHV-1 promoter consists of 5′ UTR of *oshv117* and *oshv118* and the full CDS of *oshv118*. Within (−233 and −1) 5′-UTR of *oshv117*, common eukaryotic cis-regulatory elements TATA box and CCAAT box are located (7, 41, 62). The

presence of TATA box suggests that the transcription of *oshv117* is mediated by the host RNA polymerase II (62). The topology of *oshv117* TATA box, CCAAT box, and the transcription initiation site conforms to commonly observed patterns in eukaryotes (41). In addition, we found putative binding sites to transcription factors in mammals (HIF-1, DHFR, and MAF) and yeasts (RAP1) (37–40), which together might explain the OsHV-1 promoter activity in nonmolluscan cells.

To locate the sequence within the OsHV-1 promoter responsible for its high activity, we compared the promoter activities of its subregions. Our promoter deletion analysis results show that the activity of the OsHV-1 promoter changes gradually with its length. From these data, it is suggested that multiple sequences, rather than a single critical sequence, contribute in concert to the OsHV-1 promoter activity and that some of them are part of the *oshv118* CDS.

Although the full CDS of *oshv118* was included, there was no apparent cytotoxicity in transfected cells with the OsHV-1 promoter-driven expression vectors. In fact, *oshv118* is neither a highly transactivated nor an early expressed gene in the infected oyster (34, 35). It also lacks common transcription elements like TATA box, so it is probable that *oshv118* is silent in the transfected cells.

During the infection, viral genes are expressed in a temporal order, based on which they are classified into: (i) immediate early, (ii) early, and (iii) late genes. Immediate early genes are the first genes to be expressed, relying on host cell transcription factors, in the absence of other viral gene products (22). By this nature, strong viral promoters commonly used for genetic engineering originate in viral immediate early genes (e.g., CMV IE, opIE2, CaMV 35S, and WSSV IE1 promoters) (19–21, 23). *oshv117* is considered an immediate early gene of OsHV-1 for the following reasons. First, its promoter sequence showed high activity in transfected cells, suggesting that *oshv117* can be expressed independently in the host cell without the presence of viral transcription factors. Second, *oshv117* is one of the earliest and the most highly expressed genes after infection (35). *oshv117* has a conserved RING finger (RING1) domain, known to be involved in viral replication and reactivation (63–65). RING finger proteins function as E3 ubiquitin ligases (64), which are among the common elements in viral immediate early genes (22). At present, the function of *oshv117* upon infection is unknown.

Our promoter assay confirmed moderate promoter activity of Pmy-ef1α and the CMV IE promoter in scallop cells, which agrees with previous transfection studies on oyster embryos (12–14, 16, 17). Pmy-actβ showed negligible activity in contrast to a previous study with primary oyster cells (11). This discrepancy might be due to the instability of *my-actβ* gene expression in scallop tissues (57, 66). *my-ef1β* promoter, a newly proposed reference gene for *M. yessoensis*, was not functional either. From our data, Pmy-ef1α is the most functional endogenous promoter, consistent with the high activity of the endogenous EF1α promoter in *C. gigas* embryos (12). Meanwhile, all tested WSSV- and OsHV-1-derived promoters were not functional, except Poshv088 and Poshv117.

We found that the CMV IE promoter was active in scallop cells to some extent. The CMV IE promoter is functional in oyster hemocytes and embryos (13, 14, 16, 17), in Bge cells, the only continuous cell line in freshwater molluscs (67), and even in bacterial cells (68). Therefore, it is probable that the CMV IE promoter is capable of inducing moderate, if not optimal, transcription in a broad range of organisms and can be a good starting promoter in an attempt to deliver genetic material in a nonmodel species.

We highlight that the WSSV *ie1* promoter (Pwsv-ie1) failed to induce reporter-gene expression in scallop cells. The high activity of Pwsv-ie1 was initially found in shrimp and insect cells (24). In the recombinant baculovirus-mediated transfection, it can induce high expression of a report gene in target mammalian or fish cells as well as intermediate insect cells where the baculovirus stock is amplified (58, 59). The signal transducer and activator of transcription (STAT)-binding consensus motif is the critical component for the high promoter activity of Pwsv-ie1 in a variety of cells (69). Although STAT-like proteins were also found in bivalve hemocytes (70), the fact that Pwsv-ie1 is not active in scallop cells implies that the STAT-binding motif is not well conserved in bivalves.

To summarize, we present the establishment of *M. yessoensis* primary cell culture and successful transfection of molluscan primary cells by electroporation. From a list of scallop endo- and exogenous gene promoters, we discovered a strong promoter, termed OsHV-1 promoter, from an immediate early gene of OsHV-1. The OsHV-1 promoter has clear advantages over the previously reported promoters. First, it is 25-fold more active than the commonly used promoter in bivalve molluscs so far. Second, it is a directly active promoter in molluscan cells, not dependent on multiplicity of infection of viral vectors, allowing simple and quick transfection. The OsHV-1 promoter opens the way for gene delivery and a variety of applications in bivalve molluscs. Besides facilitating basic research on gene functions, the OsHV-1 promoter can aid transgenesis of commercially important bivalve species by enhanced transfection. For cell biology, primary cells might be transformed into proliferative cell lines by OsHV-1 promoter-driven oncogene overexpression. We believe that our approach, investigating infectious virus-derived promoters, can be reproduced for genetic research in nonmodel species. Gene delivery available in marine invertebrates will contribute to a deeper understanding of comparative physiology and evolutionary genetics, underlying their astonishing regeneration, adaptation, reproduction, and aging.

Materials and Methods

Scallop Cell Culture. The Yesso scallops (*M. yessoensis*) at 1 to 2 y old were obtained from aquaculture farms in Onagawa Bay (Miyagi), Mutsu Bay (Aomori), or Funka Bay (Hokkaido), Japan.

For heart-cell culture, the ventricle, the bright part of the heart, was excised from fresh specimens and washed with 0.22-μm-filtered artificial seawater (Instant Ocean, Aquarium Systems). The collected hearts were minced with ethanol-disinfected forceps and scissors and further rinsed with DBSS (Table 1) on a shaker (50 rpm/min) for 30 min at room temperature (RT). After removal of DBSS, minced heart fragments were digested with trypsin-EDTA solution (Table 1) for 18–21 h at 4 °C. The enzymatic digestion was stopped by adding the same volume of 10% FBS-supplemented growth medium (Table 1), and suspending cells as a result of dissociation were discarded. The digested tissue fragments were explanted in multiwell plates (Sumitomo Bakelite) or T-flasks (Nunc) and cultured at 20 °C. At the initiation of cell culture, the explants were allowed to adhere to the substrate with a small volume of growth medium (Table 1), and an appropriate volume of medium was added carefully next day (in the case of 75 cm^2 T-flask, 6 mL upon seeding, followed by 9 mL added next day). The growth medium was replaced with fresh medium every 10 d or when a pH drop was apparent. When reaching confluency, primary cells were detached by cell scraper and passaged.

For culture of hemocytes, scallop hemolymph was withdrawn directly from the ventricle using a 22-gauge needle and immediately mixed with the modified Alsever's solution (MAS) 1:1 (Table 1). Without centrifugation, hemocytes in hemolymph-MAS mixture were seeded in growth medium at 2×10^5 cells/mL, making the final composition of the medium ~2% FBS, 10% scallop hemolymph-MAS mixture (Fig. 1*H*). To prevent cell aggregation, collected

hemocytes were kept on ice before seeding. In order to examine live and dead cells in culture, cells were stained by adding 0.2 μL of SYBR Green I (Midori Green Advance, Nippon Genetics) and 5 μL of 1 mg/mL propidium iodide (Cellstain PI solution, Dojindo Laboratories) per milliliter of growth medium. Cultured cells were observed and photographed under an inverted microscope (Olympus CKX53 or Keyence BZ-X800).

Optimization of Scallop Cell Culture Conditions. To determine the basal culture conditions of scallop cells, suspending scallop heart cells resulting from dissociation with trypsin-EDTA solution for 18 h at 4 °C were used. The cells were seeded and cultured with varying conditions, and elongated cells on day 2 were counted from five randomly taken micrographs at 100× magnification for each condition ($n = 20$). The examined conditions were (i) seeding density 2×10^5 or 4×10^5 cells/mL, (ii) incubation temperature 10 or 20 °C, (iii) osmolality 900 or 1,100 mOsmol/kg, and (iv) FBS 0 or 10%. For normalization, the elongated cell count was divided by two in the groups with seeding density 4×10^5 cells/mL.

Mammalian Cell Culture. Mammalian cell lines HeLa (JCRB9004) and HEK293 (JCRB9068) were provided by JCRB Cell Bank. HeLa and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, at 37 °C in a humidified incubator with 5% CO_2 supply. HeLa was used for in vitro BrdU assays, and HEK293 was used for GFP assays with viral promoters.

BrdU Assays. BrdU was incorporated into scallop cells in vivo and in vitro. For in vivo incorporation, BrdU was injected to live scallops and immunohistochemically detected as described by Osada et al. (71). In brief, live scallops were injected with 600 μL of phosphate-buffered saline (PBS) containing 4.5 mg of BrdU (24.4 mM) (Roche). After 2 d of in vivo BrdU incorporation, the heart was fixed with 4% formaldehyde. The fixed tissue was dehydrated, embedded in paraffin, and sectioned at 6 μm thickness. After deparaffinization and rehydration, the sections were incubated in anti-BrdU antibody (mouse immunoglobulin G [IgG], Roche) 1,000-fold diluted (0.1 μg/mL) in 0.1% Tween 20-PBS (PBS-T) for 2 h at 37 °C. Subsequently, the sections were incubated in goat antimouse IgG antibody (horseradish peroxidase [HRP]) (GeneTex) 200-fold diluted (1 μg/mL) in 0.1% PBS-T for 1 h at RT.

For in vitro BrdU incorporation, scallop primary heart cells on day 7 and hemocytes on day 1 were used. HeLa cells were used as a proof of concept. Scallop heart cells and hemocytes were incubated in 1 mM BrdU for 48 h, and HeLa cells were incubated in 30 μM BrdU for 48 h. After incubation, scallop primary cells were fixed with 100% methanol prechilled at −20 °C for 10 min at RT. Next, for BrdU retrieval, the fixed cells were incubated in 2 N HCl for 20 min at RT. The cells were blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich) for 30 min at RT, followed by incubation in anti-BrdU antibody 200-fold diluted (0.5 μg/mL) in 0.1% Triton X-100-PBS (PBS-TX) overnight at 4 °C. Next day, the cells were incubated in goat antimouse IgG antibody (HRP) 200-fold diluted (1 μg/mL) in 0.1% PBS-TX for 1 h at RT. BrdU-positive cells were visualized with diaminobenzidine and H_2O_2 (Histofine DAB-3S Kit, Nichirei Biosciences), and the number of total and BrdU-positive cells was counted at 100× magnification in triplicates.

Electroporation of Scallop Heart Cells. Scallop primary heart cells cultured for 1 to 2 wk were used for electroporation. To collect adherent cells, growth medium was removed and cells were washed with growth medium without FBS. The adherent cells were collected by cell scraper (Sumitomo Bakelite), and the cell suspension was filtered through a sterile 125-μm steel mesh (Yikai Metal Products) to remove remaining explants. After centrifugation at 400 × g for 5 min at 20 °C, the cell number and viability were determined by hemocytometer and trypan blue exclusion.

Electroporation was performed using NEPA21 Electroporator and 2-mm gap electroporation cuvettes (Nepa Gene). In 100 μL of Opti-MEM (Gibco) containing 10 μg of plasmid DNA or 10 μg of mRNA, 1- to 2-wk cultured 1×10^6 scallop heart cells were resuspended. Opti-MEM, used as the electroporation buffer, was supplemented with 256.7 mg/mL sucrose to adjust its osmolality to 1,050 mOsmol/kg. With these conditions, the impedance of cell suspension was consistently in the range of 50–60 Ω. The electroporation parameters were set as follows unless stated otherwise: for the poring pulse, with pulse voltage being variable, pulse length 2.5 ms, pulse interval 50 ms, number of pulses 2, decay rate 10%, and polarity switching +; for the transfer pulse, pulse voltage 20 V, pulse length 50 ms, pulse interval 50 ms, number of pulses 5, decay rate 40%, and polarity switching +/−. The poring pulse voltage from 75 to 200 V was tested to find an optimal electroporation condition in scallop heart cells. Intact cells without electroporation or cells added with distilled water instead of DNA or mRNA were used as the control. Impedance from 60 to 38 Ω was also tested by adding +20, +40, +60, +80, and +100 μL modified Opti-MEM. In assaying temporal and DNA concentration–dependent expression, scallop heart cells were electroporated with the poring pulse 200 V and the impedance 60 Ω.

Electroporated cells were seeded in 24-well plates in 500 μL of growth medium. Immediately after electroporation, cell viability was determined by trypan blue exclusion. Luciferase assays were performed at 3 dpe for mRNA-treated cells and at 2 dpe for plasmid DNA-treated cells.

Lipofection of Scallop Hemocytes. Due to severe cell mortality after electroporation, scallop hemocytes were not amenable to cuvette-based electroporation. Thus, hemocytes were alternatively transfected by lipofection for GFP assays. Lipofection was performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. Hemocytes were incubated in 500 μL of growth medium containing 500 ng of plasmid DNA, and the DNA-containing medium was replaced at 1 dpl.

Luciferase mRNA Synthesis. Capped luciferase mRNA, composed of the luciferase CDS followed by SV40 late poly(A) signal, was in vitro synthesized with pNL1.1 (Promega) using mMessage mMachine SP6 transcription kit (Invitrogen). Since pNL1.1 vector does not contain any of the T3, T7, or SP6 RNA polymerase promoter sequences, the luciferase CDS was subcloned into our pCS2-GFP vector (72). The subcloned vector was linearized using Not I (SpeedCut, LABTAS+) and used as the template for luciferase mRNA synthesis. Molecular cloning was done using In-Fusion Snap Assembly (Takara Bio).

Expression Vectors. Viral genes and proteins in WSSV and OsHV-1 herein are described conforming to zebrafish nomenclature conventions (The Zebrafish Information Network; https://zfin.org), with the virus species name followed by ORF number for gene names as in *oshv117* and *wsv465* and for protein names as in Oshv117 and Wsv465. The putative promoter regions were indicated by "P" in the first letter, as in Poshv117 and Pwsv465.

Endogenous and exogenous putative promoter regions were cloned into pNL1.1 vector. For endogenous promoters, the genomic DNA of *M. yessoensis* from Mutsu Bay was extracted from the adductor muscle using DNeasy Blood & Tissue Kit (Qiagen). The −4,706, −3,007, and −2,221 5′ upstream regions of the start codons of *M. yessoensis actβ*, *ef1α*, and *ef1β* (herein referred to as Pmy-actβ, Pmy-ef1α, and Pmy-ef1β) were cloned into Hind III (SpeedCut, LABTAS+)-linearized pNL1.1.

For exogenous promoters, 5′ upstream regions of OsHV-1 and WSSV genes were cloned into pNL1.1. For OsHV-1-derived promoters, OsHV-1-infected Pacific oyster (*Crassostrea gigas*) larvae were generously provided by Dr. Naoki Itoh (The University of Tokyo). The genomic DNA of OsHV-1 was extracted using DNeasy Blood & Tissue Kit (Qiagen), and the presence of the OsHV-1 genome was confirmed by PCR amplification of the C2/C6 fragment in *oshv4* (73). The −1,201, −1,162, −1,017, −1,038, −1,101, and −1,069 5′ upstream regions of the start codons of *oshv027*, *oshv029*, *oshv072*, *oshv080*, *oshv088*, and *oshv117* (herein referred to as Poshv027, Poshv029, Poshv072, Poshv080, Poshv088, and Poshv117) were cloned into PCR-linearized pNL1.1. For the Poshv117 deletion analysis, pNL1.1 with −902, −587, −233, and −151 5′ upstream regions of *oshv117* were obtained by deleting some sequence from pNL1.1-Poshv117.

The DNA sequences for the *wsv-ie1* promoter and *wsv465* promoter were generated by gene synthesis (Eurofins Genomics) based on the National Center for Biotechnology Information database (56), and −502 and −475 5′ upstream regions from the *wsv-ie1* and *wsv465* (herein referred to as Pwsv-ie1 and Pwsv465) start codons were cloned into PCR-linearized pNL1.1.

For GFP assays, the luciferase CDS in the foregoing luciferase vectors used in the promoter activity assay was replaced with the EGFP CDS. We used a bacterial strain DH-5α (Nippon Gene) and Plasmid DNA Extraction Mini/MIDI Kit (Favorgen) for expression vector amplification and extraction. For PCR cleanup and gel extraction, we used NucleoSpin Gel and PCR Clean-up Kit (Takara Bio) and QIAquick Gel Extraction Kit (Qiagen). All molecular cloning was done using

In-Fusion Snap Assembly (Takara Bio). All primers used in this study and PCR settings are listed in *SI Appendix*, Table S2.

Live Cell Count. In order to normalize luminescence from luciferase-electroporated cells, the number of adherent cells was counted, regarding cell attachment as an indication that cells are in a healthy state (48). For counting, we made use of high autofluorescence of scallop heart cells. With Pmy-ef1α-, Poshv088-, or Poshv117-luciferase vectors, 1×10^6 scallop primary cells were electroporated with the poring pulse 100 V and the other conditions as aforementioned. After electroporation, 5×10^4, 1×10^5, and 2×10^5 cells were seeded per well in 24-well plates in 500 μL of growth medium and cultured for 2 d. At 2 dpe, each well was washed twice with growth medium and observed at 40× magnification under a GFP filter with long exposure time (0.67 s for Keyence BZ-X800). An area of 0.77 cm^2 of autofluorescence emitted from adherent cells was photographed from each well and run through ilastik (1.3.3) Pixel Classification + Object Classification pipeline (74) for cell counting by supervised machine learning. RLU values from the luciferase assay were normalized by cell counts, setting the count from the sham control to 1.

Sequence Analysis of the Promoter and Coding Region of Oshv117. The DNA sequences of Poshv117 and *oshv117* CDS, obtained from the OsHV-1 reference strain (GenBank Accession no. NC_005881), were analyzed using the algorithms NNPP (75), NSite (76), and Conserved Domains Database (77). Repeated and palindromic sequences were searched manually. NNPP and NSite were validated by querying Pwsv-ie1 and comparing the query results with the published analysis of Pwsv-ie1 (23). The querying parameters for NSite were set as follows: ooTFD database, minimal similarity level 80%, mean expected number 0.01, and significance level 0.99.

Luciferase Assays. For luciferase assays, 1×10^6 scallop cells were electroporated with 10 μg of luciferase vector or luciferase-encoding mRNA with 100 V poring pulse. At 2 dpe for plasmid DNA-treated cells and 3 dpe for mRNA-treated cells, growth medium was removed and cells were washed twice with growth medium without FBS to leave adherent cells only. Cells were lysed by 10-min incubation in 100 μL of 1% PBS-TX per well, and the collected cell lysates were centrifuged at 12,000 × g for 5 min to remove undigested material. Luciferase assays were performed using Nano-Glo Luciferase Assay System (Promega), following the manufacturer's instructions. Luminescence was measured by Glomax Plate Reader (Promega). The measured luminescence was normalized by the number of adherent cells in each well.

GFP Assays. GFP assays were performed on scallop heart cells, scallop hemocytes, HEK293 cells, and zebrafish embryos. The cells and embryos were transfected with Poshv088- or Poshv117-EGFP vectors, with the control vector being pCS2-GFP with the CMV IE promoter. With 10 μg of each EGFP vector, 1×10^6 scallop heart cells were electroporated with 150 V poring pulse. Then, 2×10^5 scallop hemocytes were transfected by lipofection as noted above, and 3×10^5 HEK293 cells were resuspended in 100 μL of Opti-MEM (300 mOsmol/kg) and electroporated with 10 μg of each vector with 155 V poring pulse. The other poring and transfer pulse conditions were the same as in the scallop cell electroporation. For zebrafish embryos, the blastodisc at the one- or two-cell stage was microinjected with the foregoing vectors, which were adjusted to 100 ng/μL. Microinjected embryos were incubated at 28 °C and observed at 24 hpi. To avoid misinterpretation of autofluorescence as GFP expression, GFP exposure time was set based on the autofluorescence levels in the control group.

Anti-GFP Immunofluorescence. To verify that expressed fluorescence from electroporated scallop cells was true GFP signal, scallop heart cells were stained with anti-GFP antibody by immunofluorescence. The following procedure was also carried out with HEK293 cells as a proof of concept. First, with 10 μg of EGFP vectors, 1×10^6 scallop primary heart cells were electroporated with 150 V poring pulse. After 2 d, the cells were fixed with 4% formaldehyde for 15 min at RT. Permeabilization with 0.1% PBS-TX for 20 min and blocking with 3% BSA for 30 min were done at RT. Subsequently, the cells were incubated in anti-GFP antibody (rabbit IgG, Invitrogen), 200-fold diluted (10 μg/mL) in 0.1% PBS-TX overnight at 4 °C. Next day, the cells were incubated in the secondary antibody (AlexaFluor Plus 594 donkey anti-rabbit IgG, Invitrogen), 200-fold diluted (10 μg/mL) in 0.1% PBS-TX for 1 h at RT. GFP and RFP signals were observed under a fluorescence microscope.

Statistical Analysis. All data were presented as means ± SD. Normality of data were assessed by Kolmogorov–Smirnov's test. For normally distributed data, significant difference was tested by Student's *t* test for comparison between two groups and one-way ANOVA followed by Tukey's honestly significant difference test for comparisons among three or more groups, using a significance level of $P < 0.05$. For comparison between nonnormally distributed two groups, significant difference was tested by Mann–Whitney *U* test, using a significance level of $P < 0.05$. All data were analyzed and plotted using R version 4.1.2.

Data, Materials, and Software Availability. All data are included in the manuscript and/or supporting information.

ACKNOWLEDGMENTS. We thank Naoki Itoh (The University of Tokyo) for generously providing OsHV-1-infected Pacific oyster larvae and Yasuhiko Hayakawa (Nepa Gene) for invaluable advice about electroporation conditions. We are grateful to Makoto Kanamori and Masafumi Natsuike (Hakodate Fisheries Research Institute, Fisheries Research Department Hokkaido Research Organization) and Toyokazu Hiratsuka (Tohoku University Field Science Research Station) for providing the farmed Yesso scallops used in this study. This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (grant Nos. 21K19136, 20K06222, and 20H00426), Narishige Zoological Science Award (grant no. J210001964), Genome Editing Technology Research Grant (grant no. J220000495), and the JSPS Core-to-Core Program (Advanced Research Networks) entitled "Establishment of international agricultural immunology research-core for a quantum improvement in food safety."

1. B. Goldstein, N. King, The future of cell biology: Emerging model organisms. *Trends Cell Biol.* **26**, 818–824 (2016).
2. P. Martinez *et al.*, Articulating the "stem cell niche" paradigm through the lens of non-model aquatic invertebrates. *BMC Biol.* **20**, 23 (2022).
3. G. Torres, G. Charmantier, D. Wilcockson, S. Harzsch, L. Giménez, Physiological basis of interactive responses to temperature and salinity in coastal marine invertebrate: Implications for responses to warming. *Ecol. Evol.* **11**, 7042–7056 (2021).
4. T. F. Grilo, R. Rosa, Intersexuality in aquatic invertebrates: Prevalence and causes. *Sci. Total Environ.* **592**, 714–728 (2017).
5. A. G. Bodnar, Marine invertebrates as models for aging research. *Exp. Gerontol.* **44**, 477–484 (2009).
6. E. Gosling, *Marine Bivalve Molluscs* (John Wiley & Sons, Ltd., 2015).
7. H. U. Bernard, Regulatory elements in the viral genome. *Virology* **445**, 197–204 (2013).
8. J. A. Fernández Robledo *et al.*, From the raw bar to the bench: Bivalves as models for human health. *Dev. Comp. Immunol.* **92**, 260–282 (2019).
9. V. Boulo *et al.*, Infection of cultured embryo cells of the pacific oyster, *Crassostrea gigas*, by pantropic retroviral vectors. *In Vitro Cell. Dev. Biol. Anim.* **36**, 395–399 (2000).
10. J. P. Cadoret, V. Boulo, S. Gendreau, E. Mialhe, Promoters from Drosophila heat shock protein and cytomegalovirus drive transient expression of luciferase introduced by particle bombardment into embryos of the oyster *Crassostrea gigas*. *J. Biotechnol.* **56**, 183–189 (1997).
11. J. P. Cadoret *et al.*, Transient expression assays with the proximal promoter of a newly characterized actin gene from the oyster *Crassostrea gigas*. *FEBS Lett.* **460**, 81–85 (1999).
12. C. Yue, H. Yu, H. Li, S. Du, Q. Li, The endogenous EF-1α promoter is highly active in driving gene overexpression in developing embryos of the Pacific oyster *Crassostrea gigas*. *Aquaculture* **522**, 735134 (2020).
13. K. Jin *et al.*, CRISPR/Cas9 system-mediated gene editing in the Fujian Oysters (*Crassostrea angulate*) by Electroporation. *Front. Mar. Sci.* **8**, 1–6 (2021).
14. J. Chen *et al.*, PiggyBac transposon-mediated transgenesis in the Pacific oyster (*Crassostrea gigas*)–First time in mollusks. *Front. Physiol.* **9**, 811 (2018).
15. J. K. Lu, T. T. Chen, S. K. Allen, T. Matsubara, J. C. Burns, Production of transgenic dwarf surfclams, *Mulinia lateralis*, with pantropic retroviral vectors. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3482–3486 (1996).
16. J. T. Buchanan, A. D. Nickens, R. K. Cooper, T. R. Tiersch, Transfection of eastern oyster (*Crassotrea virginica*) embryos. *Mar. Biotechnol. (NY)* **3**, 322–335 (2001).
17. A. N. Tracy *et al.*, Genome to phenome tools: In vivo and in vitro transfection of *Crassostrea virginica* hemocytes. *Fish Shellfish Immunol.* **103**, 438–441 (2020).
18. Y. Wang *et al.*, Functional characterization of Cfap206 for bivalve ciliogenesis by RNAi and CRISPR/Cas9 technologies. **9**, 1–12 (2022).
19. M. Boshart *et al.*, A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521–530 (1985).
20. D. A. Theilmann, S. Stewart, Molecular analysis of the trans-activating IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology* **187**, 84–96 (1992).
21. R. Kay, A. Chan, M. Daly, J. McPherson, Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science (80-.)* **236**, 1299–1302 (1987).
22. A. Sánchez-Paz, White spot syndrome virus: An overview on an emergent concern. *Vet. Res.* **41**, 43 (2010).
23. W. J. Liu, Y. S. Chang, C. H. Wang, G. H. Kou, C. F. Lo, Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp. *Virology* **334**, 327–341 (2005).

24. L. Lu, H. Wang, I. Manopo, L. Yu, J. Kwang, Baculovirus-mediated promoter assay and transcriptional analysis of white spot syndrome virus orf427 gene. *Virol. J.* **2**, 71 (2005).
25. A. J. Davison *et al.*, A novel class of herpesvirus with bivalve hosts. *J. Gen. Virol.* **86**, 41–53 (2005).
26. B. Morga, N. Faury, S. Guesdon, B. Chollet, T. Renault, Haemocytes from *Crassostrea gigas* and OsHV-1: A promising in vitro system to study host/virus interactions. *J. Invertebr. Pathol.* **150**, 45–53 (2017).
27. M. Awaji, A. Machii, Fundamental studies on in vivo and in vitro pearl formation–Contribution of outer epithelial cells of pearl oyster mantle and pearl sacs. *Aqua-Biosci. Monogr.* **4**, 1–39 (2011).
28. R. Ladhar-Chaabouni, W. Ayadi, E. Sahli, R. Mokdad-Gargouri, Establishment of primary cell culture of *Ruditapes decussatus* haemocytes for metal toxicity assessment. *In Vitro Cell. Dev. Biol. Anim.* **57**, 477–484 (2021).
29. M. J. Metzger, C. Reinisch, J. Sherry, S. P. Goff, Horizontal transmission of clonal cancer cells causes leukemia in soft-shell clams. *Cell* **161**, 255–263 (2015).
30. B. Rinkevich, Cell cultures from marine invertebrates: New insights for capturing endless stemness. *Mar. Biotechnol. (NY)* **13**, 345–354 (2011).
31. T. P. Yoshino, U. Bickham, C. J. Bayne, Molluscan cells in culture: Primary cell cultures and cell lines. *Can. J. Zool.* **91**, 391–404 (2013).
32. K. Nagasawa, T. Thitiphuree, M. Osada, Phenotypic stability of sex and expression of sex identification markers in the adult yesso scallop *Mizuhopecten yessoensis* throughout the reproductive cycle. *Animals (Basel)* **9**, 277 (2019).
33. S. Wang *et al.*, Scallop genome provides insights into evolution of bilaterian karyotype and development. *Nat. Ecol. Evol.* **1**, 120 (2017).
34. U. Rosani, P. Venier, Oyster RNA-seq data support the development of *Malacoherpesviridae* genomics. *Front. Microbiol.* **8**, 1515 (2017).
35. A. Segarra, N. Faury, J. F. Pépin, T. Renault, Transcriptomic study of 39 ostreid herpesvirus 1 genes during an experimental infection. *J. Invertebr. Pathol.* **119**, 5–11 (2014).
36. R. Y. Birnbaum *et al.*, Coding exons function as tissue-specific enhancers of nearby genes. *Genome Res.* **22**, 1059–1068 (2012).
37. J. A. Forsythe *et al.*, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**, 4604–4613 (1996).
38. P. J. Farnham, A. L. Means, Sequences downstream of the transcription initiation site modulate the activity of the murine dihydrofolate reductase promoter. *Mol. Cell. Biol.* **10**, 1390–1398 (1990).
39. S. Mink, E. Härtig, P. Jennewein, W. Doppler, A. C. Cato, A mammary cell-specific enhancer in mouse mammary tumor virus DNA is composed of multiple regulatory elements including binding sites for CTF/NFI and a novel transcription factor, mammary cell-activating factor. *Mol. Cell. Biol.* **12**, 4906–4918 (1992).
40. I. R. Graham, A. Chambers, Use of a selection technique to identify the diversity of binding sites for the yeast RAP1 transcription factor. *Nucleic Acids Res.* **22**, 124–130 (1994).
41. W. Bi, L. Wu, F. Coustry, B. de Crombrugghe, S. N. Maity, DNA binding specificity of the CCAAT-binding factor CBF/NF-Y. *J. Biol. Chem.* **272**, 26562–26572 (1997).
42. M. Kozak, The scanning model for translation: An update. *J. Cell Biol.* **108**, 229–241 (1989).
43. K. L. B. Borden, P. S. Freemont, The RING finger domain: A recent example of a sequence-structure family. *Curr. Opin. Struct. Biol.* **6**, 395–401 (1996).
44. M. Conkling *et al.*, Breakthrough in marine invertebrate cell culture: Sponge cells divide rapidly in improved nutrient medium. *Sci. Rep.* **9**, 17321 (2019).
45. S. de Caralt, M. J. Uriz, R. H. Wijffels, Cell culture from sponges: Pluripotency and immortality. *Trends Biotechnol.* **25**, 467–471 (2007).
46. C. Rabinowitz, B. Rinkevich, Epithelial cell cultures from *Botryllus schlosseri* palleal buds: Accomplishments and challenges. *Methods Cell Sci.* **25**, 137–148 (2003).
47. A. Barrick, C. Guillet, C. Mouneyrac, A. Châtel, Investigating the establishment of primary cultures of hemocytes from *Mytilus edulis*. *Cytotechnology* **70**, 1205–1220 (2018).
48. J. T. Buchanan, J. F. La Peyre, R. K. Cooper, T. R. Tiersch, Improved attachment and spreading in primary cell cultures of the eastern oyster, *Crassostrea virginica*. *In Vitro Cell. Dev. Biol. Anim.* **35**, 593–598 (1999).
49. X. Cai *et al.*, Establishing primary cell cultures from *Branchiostoma belcheri* Japanese. *In Vitro Cell. Dev. Biol. Anim.* **49**, 97–102 (2013).
50. J. Ma, L. Zeng, Y. Lu, Penaeid shrimp cell culture and its applications. *Rev. Aquacult.* **9**, 88–98 (2017).
51. D. R. Coombe, P. L. Ey, C. R. Jenkin, Particle recognition by haemocytes from the colonial ascidian *Botrylloides leachii*: Evidence that the *B. leachii* HA-2 agglutinin is opsonic. *J. Comp. Physiol. B* **154**, 509–521 (1984).
52. K. Kawamura, S. Fujiwara, Establishment of cell lines from multipotent epithelial sheet in the budding tunicate, *Polyandrocarpa misakiensis*. *Cell Struct. Funct.* **20**, 97–106 (1995).
53. S. J. Kleinschuster, J. Parent, C. W. Walker, C. A. Farley, A cardiac cell line from *Mya arenaria* (Linnaeus, 1759). *J. Shellfish Res.* **15**, 695–707 (1996).
54. H. Hanana *et al.*, Effect of okadaic acid on cultured clam heart cells: Involvement of MAPkinase pathways. *Biol. Open* **1**, 1192–1199 (2012).
55. E. T. Jordan, M. Collins, J. Terefe, L. Ugozzoli, T. Rubio, Optimizing electroporation conditions in primary and other difficult-to-transfect cells. *J. Biomol. Tech.* **19**, 328–334 (2008).
56. F. Lin *et al.*, Characterization of white spot syndrome virus immediate-early gene promoters. *J. Gen. Virol.* **94**, 387–392 (2013).
57. Y. Li *et al.*, Systematic identification and validation of the reference genes from 60 RNA-Seq libraries in the scallop *Mizuhopecten yessoensis*. *BMC Genomics* **20**, 288 (2019).
58. Q. Wang *et al.*, Efficient and stable delivery of multiple genes to fish cells by a modified recombinant baculovirus system. *Int. J. Mol. Sci.* **19**, 1–12 (2018).
59. H. Gao *et al.*, Efficient gene delivery into mammalian cells mediated by a recombinant baculovirus containing a whispovirus ie1 promoter, a novel shuttle promoter between insect cells and mammalian cells. *J. Biotechnol.* **131**, 138–143 (2007).
60. C. Rodgers, I. Arzul, N. Carrasco, D. Furones Nozal, A literature review as an aid to identify strategies for mitigating ostreid herpesvirus 1 in *Crassostrea gigas* hatchery and nursery systems. *Rev. Aquacult.* **11**, 565–585 (2019).
61. T. Renault, "Viruses infecting marine molluscs" in *Studies in Viral Ecology* (Wiley, 2021), pp. 275–303.
62. S. T. Smale, J. T. Kadonaga, The RNA polymerase II core promoter. *Annu. Rev. Biochem.* **72**, 449–479 (2003).
63. H. Moriuchi, M. Moriuchi, J. I. Cohen, The RING finger domain of the varicella-zoster virus open reading frame 61 protein is required for its transregulatory functions. *Virology* **205**, 238–246 (1994).
64. M. W. Ferenczy, D. J. Ranayhossaini, N. A. Deluca, Activities of ICP0 involved in the reversal of silencing of quiescent herpes simplex virus 1. *J. Virol.* **85**, 4993–5002 (2011).
65. T. Renault *et al.*, Analysis of clinical ostreid herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified fragments from three virus genome areas. *J. Virol.* **86**, 5942–5947 (2012).
66. L. Feng *et al.*, Identification of reference genes for qRT-PCR analysis in Yesso scallop *Patinopecten yessoensis*. *PLoS One* **8**, e75609 (2013).
67. G. Rinaldi *et al.*, Cytometric analysis, genetic manipulation and antibiotic selection of the snail embryonic cell line Bge from *Biomphalaria glabrata*, the intermediate host of *Schistosoma mansoni*. *Int. J. Parasitol.* **45**, 527–535 (2015).
68. A. Lewin, M. Mayer, J. Chusainow, D. Jacob, B. Appel, Viral promoters can initiate expression of toxin genes introduced into *Escherichia coli*. *BMC Biotechnol.* **5**, 19 (2005).
69. W.-J. Liu, Y.-S. Chang, A. H.-J. Wang, G.-H. Kou, C.-F. Lo, White spot syndrome virus annexes a shrimp STAT to enhance expression of the immediate-early gene ie1. *J. Virol.* **81**, 1461–1471 (2007).
70. L. Canesi *et al.*, Tyrosine kinase-mediated cell signalling in the activation of *Mytilus* hemocytes: Possible role of STAT-like proteins. *Biol. Cell* **95**, 603–613 (2003).
71. M. Osada, S. Nakamura, A. Kijima, Quantitative analysis of pattern of gonial proliferation during sexual maturation in Japanese scallop *Patinopecten yessoensis*. *Fish. Sci.* **73**, 1318–1324 (2007).
72. M. Iwaizumi, H. Yokoi, T. Suzuki, Plasmid delivery by electroporation into fish skeletal muscle for recombinant protein secretion and uptake by oocytes. *Fish Physiol. Biochem.* **46**, 1121–1130 (2020).
73. T. Renault, I. Arzul, Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: Specific viral DNA detection by PCR. *J. Fish Dis.* **24**, 161–167 (2001).
74. S. Berg *et al.*, ilastik: Interactive machine learning for (bio)image analysis. *Nat. Methods* **16**, 1226–1232 (2019).
75. M. G. Reese, Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput. Chem.* **26**, 51–56 (2001).
76. I. A. Shahmuradov, V. V. Solovyev, Nsite, NsiteH and NsiteM computer tools for studying transcription regulatory elements. *Bioinformatics* **31**, 3544–3545 (2015).
77. S. Lu *et al.*, CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Res.* **48** (D1), D265–D268 (2020).