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Short communication

Genome to phenome tools: *In vivo* and *in vitro* transfection of *Crassostrea virginica* hemocytes

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ABSTRACT

The sequencing of the *Crassostrea virginica* genome has brought back the interest for gene delivery and editing methodologies. Here, we report the expression in oyster hemocytes of two heterologous expression vectors under the CMV promoter delivered with dendrimers. Expression was monitored using confocal microscopy, flow cytometry, and immunofluorescence assay. *C. virginica* hemocytes were able to express the green fluorescence protein and *Crassostrea gigas* vascular endothelial growth factor under CMV viral promoter both *in vivo* and *in vitro*. These results provide the bases for interrogating the genome and adapting genome editing methodologies.

Oysters are considered ecosystem builders by cycling particulate matter and phytoplankton, hence stabilizing fragile estuary nutrient cycles and fueling higher trophic levels [1,2]. Additionally, oyster aquaculture is a valuable economic resource for coastal areas [3]. The development of aquaculture is often accompanied by disease outbreaks resulting in financial losses and disturbance of the marine ecosystem [4–8]. Hemocytes represent the mainline of defense against pathogens [9–12] and are also involved in many other physiological events, including nutrient transportation, detoxification, and wound repair (reviewed in Ref. [13]). The protozoan parasite *Perkinsus marinus* responsible for 'Dermo' disease [14]. *P. marinus* uses the galectin CvGal1 to enter the hemocytes [10,12,15,16] and takes advantage of transepithelial migration of mucosal hemocytes to gain access to the circulating hemolymph [17,18]. The understanding of the role of hemocytes in these processes has been hindered by the lack of a genetically tractable system. A well-annotated genome is essential for a genetically tractable system to generate mechanistic hypotheses and a genetic delivery system to test those hypotheses at a cellular level. With the *Crassostrea virginica* genome (C_virginica-3.0; GCF_002022765.2) already available [19], a robust genetic delivery system would provide a unique opportunity for moving from genome to phenome.

The pioneering work for delivering genetic material into oyster primary cell cultures and embryos took place more than 20 years ago using heterologous promoters and available commercial electroporation reagents. Lipofection-mediated transfection of *C. gigas* heart primary cell cultures was achieved under transcriptional control of several heterologous promoters (*Drosophila hsp70* promoter, cytomegalovirus, CMV, and SV40 early promoters) [20], and the upstream flanking region of the *C. gigas* [21]. Transfection of 3 h-postfertilization *C. virginica* embryos has been achieved using SV40 and the immediate-early promoter from human CMV by electroporation [22]. Whereas, adult oysters injected with the commercial plasmid *p*S65T-C1 using dendrimers resulted in fluorescent hemocytes [22].

In this study, we investigated *in vivo* and *in vitro* transfection and expression of heterologous plasmids in the hemocytes of the eastern oysters (*C. virginica*). We employed a chemical delivery approach based on Buchanan et al. [22] using *p*max-GFP (Lonza, Walkersville, MD, USA), a plasmid which has CMV promoter driving GFP expression. Commercially purchased adult oysters from the Damariscotta River Estuary (DRE) (Maine, USA) were notched at the posterior end on the dorsal side of the shells using a rotary tool (Dremel, Racine, Wisconsin, USA) equipped with a carbide rotary burr, and maintained in DRE water for 1 day to recover.

For *in vivo* studies, hemolymph was withdrawn from six oysters using a 1-ml insulin syringe with a 19G needle containing 100 µl of Alsever's solution (Alfa Aesar, Tewksbury, MA). Samples were centrifuged at 4 °C and 1000 × *g* for 10 min, and the supernatants (serum) were pooled and filter sterilized using 0.2/0.8 syringe filters with

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Supor® membrane (Pal Corporation, CA, USA). For each oyster, 5 μg of dried *p*max-GFP (Lonza, Benicia, CA, USA) were dissolved in 33 μl of pooled oyster serum and 67 μl of SuperFect (QIAGEN, Germantown, MD, USA) (ratio 1:2). The *p*max-GFP, SuperFect, and plain serum from the pooled oyster hemolymph were used as controls. In all the cases, each oyster received 100 μl of the transfection cocktail in the adductor muscle using a syringe. The oysters were incubated for 3 h at room temperature on the bench before returning them to the aerated buckets with filtered seawater at 12 °C. The GFP expression after withdrawing hemolymph (200 μl) from the adductor muscle as above, was monitored using both confocal microscopy (LSM-700, Carl Zeiss, Pleasanton, CA, USA) and flow cytometry (ZE5 Cell Analyzer, Bio-Rad, Hercules, CA, USA) at days 1, 4 and 9 respectively. A similar set of experiments were performed using *p*CgVEGF encoding an HA-tagged *C. gigas* vascular endothelial growth factor (VEGF) and IRES (Internal Ribosome Entry Site)-GFP under CMV control (*p*CgVEGF-HA-IRES-GFP). *C. gigas* VEGF was cloned and fusion tagged with the HA antigen by PCR (primers: F: 5′ TAC CGT GAG CTC ATG GAC ATA CTA ATG CGA GCT 3'; R: 5′ TAA TGT CCG CGG TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GGG AAA TTC AGG ACC AGC GGC 3′). Full-length CgVEGF-HA was cloned into the *p*IRES-2-GFP vector (Takara Bio USA, Mountain View, CA, USA) using *Sac*I and *Sac*II restriction sites engineered into the primers to create *p*CgVEGF-HA-IRES-GFP. The sequence was deposited in GenBank (MT226487). In addition, we performed experiments *in vitro* using oyster hemocytes from individual oysters and both sets of plasmids. In this case, hemolymph samples were centrifuged at 4 °C and 1000 g for 10 min, and the cell pellet resuspended in the cocktail containing each of the plasmids resuspended in the corresponding oyster serum and SuperFect (ratio 1:2 as above). The resuspended cells were transferred to a CytoOne 12-well plate (USA Scientific, Ucala, FL, USA) and maintained in a 12 °C incubator. Fluorescence was monitored as stated above after the hemocytes were detached from the bottom of plate using a cell scrapper (Biologix, Southern Labware, GA, USA). For the immunofluorescence assay (IFA), cells were incubated on ice to allow hemocytes to adhere to NunC Lab-Tek II chambered coverglass (ThermoScientific, Waltham, MA, USA). The hemocytes were then fixed with 3% paraformaldehyde (PFA) overnight at 4 °C then washed 3 times using PBS to remove excess PFA. For co-localization studies, 1:5000 diluted primary rabbit monoclonal anti-HA antibodies (Cell signaling, Danvers, MA, USA), and 1:10,000 diluted mouse monoclonal anti-GFP antibodies were used. Briefly, cells were incubated for 1 h at 37 °C in a humid chamber, then washed three times with PBS to remove unbound primary antibody. To confirm the expression and localization of CgVEGF using anti-HA, hemocytes were incubated with Alexa 568 conjugated anti-IgG rabbit antibody (Molecular Probes, Eugene, OR). For GFP expression confirmation, hemocytes labeled with anti-GFP were incubated with rhodamine isothiocyanate (RITC) conjugated anti-mouse IgG secondary antibodies (Southern Biotech, Birmingham, AL, USA). Following incubations, the cells were washed three times with PBS and once in distilled water to remove unbound secondary antibodies. Preimmunized rabbit and mouse serum were used as controls. ProLong Diamond Antifade mountant supplemented with 4′, 6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) was used to mount the coverglass on to the slide. For confocal microscopy images, Zeiss LSM-700 laser scanning confocal microscope with Alexa 488, 555, and 568 filters were used. Images were merged using ImageJ software [23], and Adobe Photoshop CC (Adobe Inc., Mountain View, CA, USA) was utilized for 600 DPI conversion. In both *in vivo* and *in vitro* experiments, we were able to detect fluorescent cells as early as 1-day post-transfection, and expression was continuous for 9 days after oyster receiving transfection cocktail. In both *in vitro* and *in vivo* transfections, hemocytes/oysters transfected with *p*max-GFP and *p*CgVEGF-HA-IRES-GFP exhibited the green fluorescent protein spread in the cytoplasm of the hemocytes as early as 24 h post-transfection, and punctate GFP accumulation were observed 4 days post transfection (Fig. 1A *in vitro* and *in vivo* confocal). In *in vivo* and *in vitro* experiments, we have seen higher levels of GFP expression in the cells of diameter 5 μm and 10 μm respectively. Cells of diameter greater than 15 μm had either reduced or no fluorescence (not shown). Suggesting hyalinocytes and intermediate cells were efficient in gene expression in comparison to the granulocytes.

We evaluated transfection efficiency of cells expressing GFP using flow cytometry; log of forward scatter 488/10 area (x-axis) *vs.* log of 525/35 (GFP) - area (y-axis) were used to differentiate autofluorescent cells and identify GFP expressing cells when excited with 488 nm laser light (Fig. 1B Panel: *in vitro* and *in vivo* highlighted in green). Among 3 technical replicates of *in vitro* samples transfected with *p*max-GFP, an average of 0.07% of GFP cells was detected (Fig. 1C *in vitro* panel). The hemocytes transfected with *p*CgVEGF-HA-IRES-GFP exhibited the transfection efficiency of 0.13% (Fig. 1C *in vitro* panel). With both plasmids, the fluorescence was above the controls. The transfection efficiency in the *in vivo* experiments were of 0.010% with *p*max-GFP (Fig. 1C *in vivo* panel) and 0.012% with *p*CgVEGF-HA-IRES-GFP (Fig. 1C *in vivo* panel) in the *in vivo* experiments. As we observed higher transfection efficiency in the *in vitro* experiment, *in vitro* cells were used in the IFA. IFA using anti-HA rabbit monoclonal antibodies confirmed the successful synthesis of the CgVEGF growth factor and its localization in the cytoplasm of the hemocytes (Fig. 1D, panel i). Additionally, anti-GFP mouse monoclonal antibodies confirmed the synthesis of GFP in the *in vitro* experiments transfected with *p*max-GFP (Fig. 1D, panel ii).

Previous transfection studies in *C. virginica* (*in vivo*) and embryos (*in vitro*) [22] provided a viable method for hemocyte withdrawal and maintenance, as well as a plasmid with CMV promoter as a reliable tool for the gene expression. Here we utilized two plasmids with CMV promoter to test gene expression in our optimized protocol. To target highly active hemocytes, we performed transfections in oysters that were recovered for 1-day post notching. We observed the successful expression of GFP both *in vivo* and *in vitro* as early as 24 h post-transfection in comparison to the 4 days post-transfection, as reported previously [22]. To our knowledge, we are reporting for the first time the successful transfection of *in vitro* primary hemocyte cultures and compared this with *C. virginica* oyster embryos. We took advantage of the polycistronic plasmid *p*CgVEGF-HA-IRES-GFP cloned with *C. gigas* vascular endothelial growth factor to demonstrate for the heterologous gene expression in *C. virginica* hemocytes for the first time. Though the *in vitro* transfection efficiency in our method is low, this can be addressed by standard optimization protocols in the future. With many moving parts (oyster intraspecific variability both in terms of cell types and number, heterologous plasmid, origin of the cells used), it is difficult to identify what is mainly responsible for the low efficiency. We think that the lack of a hemocyte specific homologous expression plasmid with a strong promoter is one of the main hurdles to achieve higher transfection efficiency. An oyster cell line would reduce most of the variability seen in these experiments. Here we targeted hemocytes because they can be quickly and non-lethally withdrawn from the adductor muscle [20,21]; however, the same approach can be applied to cardiac, mantle, gill cells that can be obtained by enzymatical disaggregation [24–26].

We further performed *in vivo* transfections with SuperFect and two heterologous plasmids under the SV40 promoters: SV40 basal promoter-GFP-IRES-AP (Plasmid #18807), *p*MLS-SV40-EGFP (Plasmid #46919) (Addgene, Watertown, MA, USA). The pattern of the fluorescent protein expression was similar to the *p*max-GFP data, as discussed above (data not shown). Physical plasmid delivery using electroporation of the hemocytes also resulted in a small number of fluorescent cells (data not shown). Compared to *in vitro* electroporation, a dendrimer-based chemical method is the most convenient protocol since it does not require special equipment. These results open the door to design and test homologous expression plasmids necessary for unraveling the oyster's biology, developing cell lines, and accessing tools for editing the oyster genome [13]. This is especially relevant now that

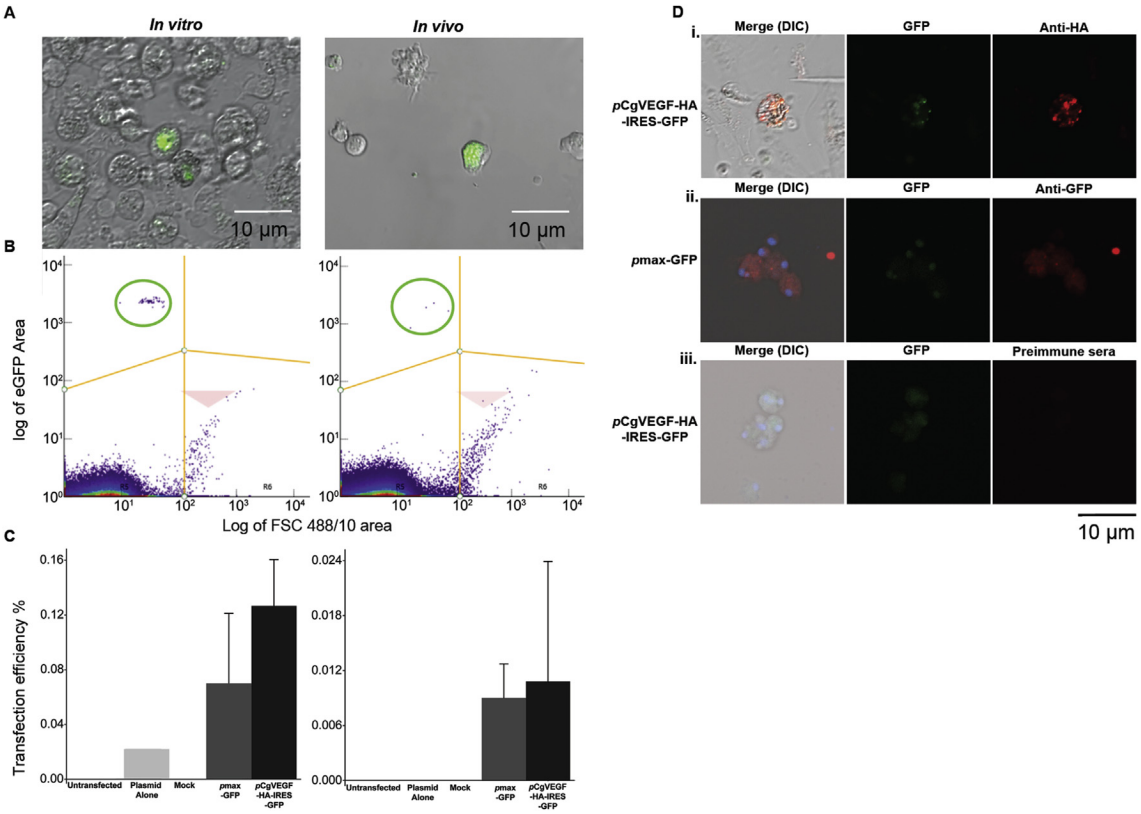

Fig. 1. Expression of heterologous genes *in vivo* and *in vitro* primary cultured oyster (*Crassostrea virginica*) hemocytes. A: Green fluorescent cells confirming GFP expression in Hemocytes (*in vitro*) and *C. virginica* oyster (*in vivo*) transfected with *p*max-GFP Scale bar, 10 µm. B: Scatter plots showing the 525/35 GFP positive cells collected 4 days post transfection indicated with green circle in *in vitro* and *in vivo* experiments. C: Bar graphs showing the % of transfection efficiency of *p*max-GFP (dark grey bar) and *p*CgVEGF-HA-IRES-GFP (black bar) in the *in vitro* and *in vivo* experiments. D: Indirect immunofluorescence analysis of hemocytes transfected with (i) *p*CgVEGF-HA-IRES-GFP showing GFP expression (GFP panel) and synthesis of CgVEGF confirmed using anti-HA monoclonal antibodies (Red panel). (ii) *p*max-GFP showing GFP expression and the GFP synthesis using anti-GFP mouse monoclonal antibodies (Red) transfected. (iii) Preimmunized sera did not show reactivity with HA-tag. Data is representative of n = 4 experimental replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CRISPR/Cas9 gene editing in *C. gigas* has also recently been achieved using ribonucleoprotein complexes and gRNA microinjected into embryos [27].

CRediT authorship contribution statement

Adrienne N. Tracy: Validation, Formal analysis, Investigation, Writing - original draft. **Raghavendra Yadavalli:** Conceptualization, Formal analysis, Writing - original draft, Visualization, Supervision. **Kiara S. Reed:** Methodology, Validation, Investigation, Writing - review & editing. **Rahul Parnaik:** Methodology, Validation, Writing - review & editing. **Nicole J. Poulton:** Formal analysis, Writing - review & editing, Supervision. **David Bishop-Bailey:** Conceptualization, Writing - review & editing. **José A. Fernández Robledo:** Conceptualization, Formal analysis, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

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