INVESTIGATION OF THE MOLECULAR MECHANISMS UNDERLYING ESTROGEN-MEDIATED INDUCTION OF VITELLOGENIN GENE EXPRESSION IN THE SYDNEY ROCK OYSTER, SACCOSTREA GLOMERATA

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A thesis submitted for the degree of Doctor of Philosophy
University of Newcastle
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February, 2017
DEDICATION

To the memory of my beloved father, Quy Tran (1943-2014), who worked very hard for his whole life to support for my education.
To my great husband, Nam Mai, for his true love and support.
DECLARATIONS

Originality

The work described in this thesis was conducted between March 2012 and February 2017 at the School of Environmental and Life Sciences, the University of Newcastle and Port Stephens Fisheries Institute, NSW Department of Primary Industries. The work was performed and analysed by myself unless stated otherwise.

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary Institutions and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository, subject to the provisions of the Copyright Act 1968.

Thesis by Publication

I hereby certify that the work embodied in this thesis contains a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications. As a primary author of all publications included, I, Thi Kim Anh Tran, was involved in the planning and performing of all experiments, data analyses and preparation of all manuscripts. The co-authors assisted with planning the experiments, providing experimental materials, interpreting data, revising the manuscripts and giving general support.

Thi Kim Anh Tran
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Drs. Richard Yu and Geoff MacFarlane from the School of Environmental and Life Science, the University of Newcastle for their thorough guidance and significant contributions to this thesis. Richard provided knowledge about molecular biology and lab training. He also assisted in preparing all manuscripts and getting papers ready for publication. Geoff provided support, guidance and reviews for this thesis and all manuscripts. Words are not enough to express my gratitude to them. Also, I am grateful to friends from the Ecotoxicology and Ecology lab, especially Dr. Thanvapon Yingprasertchai, for his care since I started my lab work.

I received invaluable assistance from numerous people in different places and in different ways: Dr. Wayne A. O’Connor (Port Stephens Fisheries Institute, NSW Department of Primary Industries) provided oysters, facilities and his professional advice on performing tank exposure experiments. Also from Port Stephens Fisheries Institute, Kyle Johnston provided help in collecting farmed oysters and operating the facilities for the exposure experiments. Dr. Richard Yuen Chong Kong (City University of Hong Kong) kindly assisted in DNA sequencing. Dr. John Abbenante, Applications and Technical Specialist from BMG Labtech Pty Ltd provided technical support for the operation of the FeraStar FS microplate reader. Mr. Kim Colyvas and Mr. Nic Croce, UoN Statistical Consultants, assisted in statistical analysis. I also could not expand my knowledge and research network without the travel grants provided by SETAC-AU and -EU.

Last, but certainly not least, the biggest thank you goes to my family and friends who have always been an enormous support to me. A special thanks to my husband, Nam Mai, for his true love, support and encouragement.

The PhD study was sponsored by a joint scholarship between the Vietnamese Government and The University of Newcastle.

Thi Kim Anh Tran
LIST OF PUBLICATIONS, PRESENTATIONS AND AWARDS

Publications


Conference presentations


- Molecular cloning and characterization of an estrogen receptor gene in the Sydney rock oyster (Saccrostrea glomerata). The fourth SETAC Young Environmental Scientists (YES) meeting, Petnica Science Centre, Serbia, March 2015. Poster high-light presentation.

Awards:

• Research Higher Degree Scholarship granted by VIED, Vietnam and the University of Newcastle, Australia (2012-2016).

• 2016 Post Graduate Research Publication Award, Society of Environmental Toxicology & Chemistry – Australasia (SETAC-AU)

• Student Travel Award, SETAC-AU for 2016 conference, Hobart, October, 2016.

• Student Travel and Accommodation Award, SETAC-EUROPE for the 4th SETAC Young Environmental Scientists (YES) meeting. Petnica Science Centre, Serbia, March 2015.

• Student Travel Award, SETAC-AU for the 9th SETAC Asia/Pacific conference. Adelaide, September, 2014.
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LIST OF ABBREVIATIONS

ALP: Alkali labile phosphates

ARC: Australian research council

AP1: Activating protein-1

BPA: Bisphenol A

CpG: Cytosine-phosphate-Guanine

cDNA: Complementary deoxyribonucleic acid

CREB: cyclic AMP response element-binding protein

DES: Diethylstilbestrol

DNA: Deoxyribonucleic acid

E1: Estrone

E2: 17β-estradiol

E3: Estriol

EE2: 17α-ethinylestradiol

EDCs: Endocrine disrupting chemicals

ECs: Estrogenic compounds

ELISA: Enzyme-linked immunosorbent assay

EMSA: Electrophoresis mobility shift assays

EPA: Environmental protection agency

ER: Estrogen receptor

LBD: Ligand binding domain

DBD: DNA binding domain
ERE: Estrogen responsive element
mRNA: Ribonucleic acid messenger
miRNA: microRNA
NSW: New South Wales
NP: Nonylphenol
OP: Octylphenol
PCB: Polychlorinated biphenyls
PCR: Polymerase Chain Reaction
qT-PCR: Quantitative Real-time Polymerase Chain Reaction
RNAi: RNA interference
TFs: Transcription factors
WWTP: Wastewater treatment plant
Vtg: vitellogenin
LPD_N: Lipoprotein N-terminal domain
DUF1943: Domain of unknown function 1943
VWD: Von Willebrand factor type D domain
E2-BSA: Estradiol-bovine serum albumin
Sp1: Stimulating protein -1
SgER: *Saccostrea glomerata* estrogen receptor
SgVtg: *Saccostrea glomerata* Vitellogenin
SRO: Sydney rock oyster
ABSTRACT

Environmental estrogens are known to interfere with normal development and reproduction in a wide range of marine invertebrates. Previous studies have developed an oyster biomonitor to indicate the presence of estrogenic compounds in marine environments through exploiting the induction of the egg yolk protein precursor vitellogenin (Vtg). Despite this advance, the mechanism through which estrogens exert their action on Vtg gene expression, in particular the functional role of the estrogen receptor (ER), is currently unknown. In an attempt to fill this knowledge gap, the present research aims to isolate the genes encoding Vtg (sgVtg) and ER (sgER) from the Sydney rock oyster *Saccostrea glomerata* and investigate different potential mechanisms contributing to estrogen mediated induction of sgVtg.

Our results indicated that the deduced protein of sgVtg is substantially longer than those of the other oyster Vtgs reported so far and contains all the conserved domains as found in other marine molluscs. The sgVtg promoter contains multiple putative half-EREs which are closely spaced, implying that they may function as an estrogen response unit (ERU) to interact with ER. In line with the potential involvement of ER in sgVtg regulation, the induction of sgVtg mRNA expression in ovarian explants was shown to be abolished by the ER antagonist ICI 182, 780. Considering that the vertebrate-like ER so far reported in molluscs lacks estrogen-binding ability, this finding supports the requirement of a novel estrogen-binding receptor for gene activation.

The sgER cDNA is predicted to encode a 477-amino acid protein, which contains a DNA binding domain (DBD) and a ligand binding domain (LBD) conserved among vertebrate and invertebrate ERs. Comparison of the sgER LBD sequence with those of other ligand-dependent ERs indicated that the sgER LBD is degenerate at several conserved residues critical for ligand binding and receptor activation. Its inability to bind estrogens was then confirmed by a ligand binding assay using fluorescent-labelled E2 and purified sgER protein. The 5′-flanking region of sgER contains three putative 1/2EREs and several other putative elements for ER-interacting transcription factors, suggesting potential autoregulation of sgER expression. sgER mRNA is ubiquitously expressed in various tissues, with the highest expression level observed in the ovary where sgVtg is highly
expressed. Functional analyses, including luciferase gene reporter assays, point mutation of $\frac{1}{2}$ EREs and an electrophoretic mobility shift assay (EMSA), confirmed that sgER binds and activates the $sgVtg$ promoter through $\frac{1}{2}$EREs. In addition, $sgER$ mRNA was significantly upregulated following in vitro and in vivo exposure to E2 and the enhancing effect of E2 on $sgER$ expression was abolished by co-treatment with the specific ER antagonist ICI 182, 780 in vitro. These findings support the presence of a novel estrogen-binding receptor in S. glomerata.

To elucidate whether estrogens modulate $sgVtg$ and $sgER$ expression at the epigenetic level, we assessed the DNA methylation levels of a 5’ intragenic CpG island in $sgVtg$ and a promoter CpG island in $sgER$ in ovaries after E2 exposure in vivo. Bisulfite sequencing revealed that both of these CpG islands are hypomethylated in both control and E2-treated oysters. Neither significant differential DNA methylation nor correlation between DNA methylation and mRNA levels was observed for either $sgVtg$ or $sgER$.

Overall, the findings from this research provides new molecular insights into how environmental estrogens regulate Vtg expression in marine molluscs and lays the foundation for further research into the mechanism of action of estrogenic compounds on molluscan vitellogenesis.
This thesis by publication outlines the body of work that I have contributed through a series of scientific publications towards the investigation of the molecular mechanisms underlying estrogen-induced Vtg gene expression in *S. glomerata*.

Chapter one starts with an introduction, which overviews the rationale for the entire research project. This is followed by a literature review, which presents the current understanding of various topics related to this research, including the concepts of endocrine disrupting chemicals (EDCs), estrogenic compounds (ECs) and the biomarker of exposure to ECs, vitellogenin (Vtg), and the potential molecular mechanisms underlying Vtg induction in aquatic vertebrate and invertebrate species. Finally, the review sets out the research questions, hypotheses and specific aims of this research.

Chapter two consists of the first published journal article generated from this research (Tran et al., 2016, *Aquatic toxicology* 174, 146‒158). This paper reports on the molecular isolation of the cDNA and genomic sequences of *sgVtg* and characterisation of its expression through *in vitro* and *in vivo* exposure to E2, treatment with the ER antagonist ICI 182, 780 and DNA methylation analysis of a 5' intragenic CpG island. This study highlighted the crucial role of a novel estrogen-binding receptor in the transcriptional control of a molluscan Vtg gene.

Chapter three consists of the second publication generated from this research (Tran et al., 2016, *Aquatic toxicology*, 179, 82‒94). This paper reports on the molecular isolation of the cDNA and promoter sequences of *sgER*, analytical evaluation of the estrogen-binding ability of the purified sgER protein and characterisation of *sgER* expression through *in vitro* and *in vivo* exposure to E2, treatment with the ER antagonist ICI 182, 780 and DNA methylation analysis of a 5' intragenic CpG island. This study highlighted the crucial role of a novel estrogen-binding receptor in the transcriptional control of a molluscan Vtg gene.
vitro and in vivo exposure to estrogen, treatment with ICI 182, 780 and DNA methylation analysis of a promoter CpG island. This study confirmed that sgER is a non-estrogen binding receptor and again supported the role of a novel estrogen-binding receptor in estrogen signalling in molluscs.

Chapter four consists of a manuscript accepted for publication in the peer-review journal *Marine Pollution Bulletin*. It presents the functional characterisation of the *sgVtg* promoter using the following molecular assays: (1) luciferase reporter gene assays to assess the transactivation potential of sgER on the *sgVtg* promoter and its estrogen dependence; (2) point mutation of the putative ½EREs residing within the *sgVtg* promoter to evaluate their functional role in transactivation; and (3) an electrophoretic mobility shift assay (EMSA) to determine the binding of sgER to the putative ½EREs. This work affirmed the functional role of a constitutive actively ER in transactivating the Vtg gene promoter in molluscs.

Chapter five is a general discussion which summarises the main results obtained from Chapters two to four and proposed future prospects for research.