Elsevier Editorial System(tm) for Gene
Manuscript Draft

Manuscript Number: GENE-D-14-02408R1

Title: Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two Mytilus species

Article Type: Research Paper

Keywords: mussel; reproduction; endocrine disruption; autoregulation

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Manuscript Region of Origin: JAPAN

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Opposed Reviewers:

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Vertebrate-like sex steroid hormones have been widely detected in mollusks, and numerous experiments have shown the importance of steroids in gonad development. Nevertheless, their signaling pathways in invertebrates have not been uncovered yet. Steroid receptors are an ancient class of transcription factors with multiple roles in not only vertebrates but also invertebrates. Estrogen signaling is thought to have major roles in mollusk physiology, but the full repertoire of estrogen receptors is unknown. We presented the successful cloning of two novel forms of estrogen receptor-like genes. These receptors are present in two closely related species of *Mytilus*: *Mytilus edulis* and *Mytilus galloprovincialis*, commonly known and widely distributed sentinel species. Our phylogenetic analysis revealed that one of these receptors is an estrogen receptor (ER) and the other one is an estrogen-related receptor (ERR). Studies of expression analysis showed that both receptor mRNAs were localized in the oocytes and follicle cells in contact with developing oocytes in the ovary and Sertoli cells in the testis, and in the ciliated cells of the gill. In addition, we have evidence that one (ER) of these may have a capacity to autoregulate its own expression in the gonadal cells by estrogen (E_2) and that this gene is responsive to estrogenic compounds.

Highlights:

- We cloned two forms of full-length ER-like genes from two *Mytilus* species.
- One is an ER and the other one is an ERR.
- Possessing an ER and an ERR may be a fundamental system for most mollusks.
- Their mRNAs were both detected in the oocytes, follicle cells, and Sertoli cells.
- ER transcription may be autonomously upregulated in the gonadal cells by E_2 treatment.

Abbreviations list

ER; estrogen receptor, ERR; estrogen-related receptor, SR; steroid hormone receptor, E_2; estradiol-17ß, T; testosterone

Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two *Mytilus* species.

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Running title:
Molecular characterization of *Mytilus* estrogen receptors

This ms has 17 pages, 5 figures

Abstract

Vertebrate-like sex steroid hormones have been widely detected in mollusks, and numerous experiments have shown the importance of steroids in gonad development. Nevertheless, their signaling pathways in invertebrates have not been uncovered yet. Steroid receptors are an ancient class of transcription factors with multiple roles in not only vertebrates but also invertebrates. Estrogen signaling is thought to have major roles in mollusk physiology, but the full repertoire of estrogen receptors is unknown. We presented the successful cloning of two novel forms of estrogen receptor-like genes. These receptors are present in two closely related species of *Mytilus*: *Mytilus edulis* and *Mytilus galloprovincialis*, commonly known and widely distributed sentinel species. Our phylogenetic analysis revealed that one of these receptors is an estrogen receptor (ER) and the other one is an estrogen-related receptor (ERR). Studies of expression analysis showed that both receptor mRNAs were localized in the oocytes and follicle cells in contact with developing oocytes in the ovary and Sertoli cells in the testis, and in the ciliated cells of the gill. In addition, we have evidence that one (ER) of these may have a capacity to autoregulate its own expression in the gonadal cells by estrogen (E_2) and that this gene is responsive to estrogenic compounds.

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1. Introduction

Many of controversial topics still persist on the knowledge of invertebrate steroid hormones with their receptors even though numerous studies have been conducted for more than five decades (Hultin et al., 2014). These arguments can be attributed to the unresolved issue that there has never been concrete evidence for the presence of endogenous sex steroid hormones in invertebrates despite evidence indicating the possible presence of invertebrate estradiol-17β (E_2) (Reis-Henriques et al., 1990; Zhu et al., 2003). In mollusks, Scott (2012; 2013) concerned the interference of a non-specific assay for detecting mollusk steroids based on the detection method for vertebrate-type steroids because no conclusive evidence exists for endogenous steroid hormones in any mollusk. Nevertheless vertebrate-type steroids that respond along with their reproductive cycle or exposure to endocrine disrupting chemicals (EDCs) (Scott, 2013) have been detected in many mollusk species (Janer and Porte, 2007; Matsumoto et al., 2007). Janer and Porte (2007) systematically summarized the possible sex steroidogenic pathways in several invertebrates (i.e. echinoderms, crustaceans, and mollusks). In the recent review, Giusti and Joaquim-Justo (2013) proposed that the endogenous pathway of steroid hormone synthesis (i.e. esterification) and biologically-active vertebrate-like steroid hormones exist in at least three mollusk classes: Bivalvia, Gastropoda, and Cephalopoda. Nevertheless, there has been no firm evidence of the presence for functional receptors to vertebrate-like steroids and that vertebrate-like steroids function on mollusk endocrine systems via their specific receptors (Scott, 2012).

Intriguingly, Thornton's lab has investigated the evolutionary divergence in physiological activity of steroid hormone receptors (SR) especially for lophotrochozoan ERs with their ligand-specific activity (Thornton et al., 2003; Keay and Thornton, 2009; Eick and Thornton, 2011). A recent study combining evolutional genetics and protein structure from his group delivered an interesting insight in lophotrochozoan SR biology including ERs (Bridgham et al., 2014). They reported that annelid ERs are activated by the vertebrate-type estrogen (E_2) (Keay and Thornton, 2009), whereas several other mollusk ERs (e.g. Aplysia (Thornton et al., 2003), land snail, limpet, marine snail (Kajiwara et al., 2006), octopus (Keay et al., 2006) and oyster (Matsumoto et al., 2007)) are constitutively active transcription receptors. Using X-ray crystallography to investigate the structure, they also found a

clue to the regulatory mechanism in the constitutive transcriptional activity of ER by showing that structural variation of the ligand-binding pocket filled with bulky residues preventing ligand occupancy in oyster ER (Bridgham et al., 2014).

In terms of molecular evolution of ER, vertebrates are known to have multiple ERs and estrogen-related receptors (ERRs) as part of their SR repertoire. Estrogen receptors (ERs) are a group of nuclear receptor superfamily of steroid hormones classified as ligand-activated transcription factors that modulate gene expression. Whereas ERRs are orphan nuclear receptors possessing similar structure and sequence similarity to ERs. However, ERRs bind to not only estrogen response elements but also their own response elements (ERR response elements), and then modulate transcription but are not activated by estrogens (Horard and Vanacker, 2003). Both ERs and ERRs are widely distributed in different tissue types, however there are some notable differences in their expression patterns (Bookout et al., 2006). The ERs and ERRs are found in diverse species throughout the animal kingdom. Evolutionary genetics has revealed that two distinct subtypes of nuclear ERs (ERα and ERβ) act in vertebrates including mammals (Keaveney et al., 1991) and single ER is currently identified in a wide variety of invertebrates such as nematodes (Mimoto et al., 2007), annelids and mollusks (Eick and Thornton, 2011). Whereas, the mammals possesses three different subtypes of ERR (ERR-α, ERR-β, and ERR-γ) in their genome (Horard and Vanacker, 2003). In invertebrates, the ERRs are not very well understood. Baker (2008) reported that the sequenced *Trichoplax* genome has an ERR, but no ER. Furthermore, additional BLAST searches found ERRs in other three lophotrochozoan transcriptomes: an annelid worm, a leech, and a snail. Whereas invertebrate-like ERs exist in worm and snail, but not in leech. Another report showed that the freshwater snail *Marisa cornuarietis* has two transcripts that encode an invertebrate-like ER and an ERR (Bannister et al., 2007). However, there has not been enough examples which verify the presence of both invertebrate-like ER and an ERR in mollusks.

In the present study, we focused on two *Mytilus* species: *Mytilus edulis* and *Mytilus galloprovincialis* as commonly known and widely distributed sentinel species which are potentially susceptible to EDCs. We previously reported that a significant increase of *vitellogenin* (VTG) and ER2 transcripts was caused by estrogen exposure in *M. edulis* at the early stage of gametogenesis, but not at mature stages (Puinean et al., 2006; Ciocan et al., 2010). Nevertheless in these *in vivo* studies, invertebrate-like

ER and ERR have not been fully investigated in *Mytilus* species yet from the point of view of evolutionary genetics and molecular characterization. To build the basic knowledge of invertebrate-like ER in bivalves, this study aimed to clone the full-length coding cDNA sequence of both invertebrate-like ER and ERR from two *Mytilus* species, and to characterize their molecular behavior, particularly gene expression responses to estrogen exposure.

2. Materials and methods

2.1. Animals

Mytilus edulis was sampled from Brighton, UK and provided from Indian Point Mussel Farm, Canada in the summer of 2010, and *Mytilus galloprovincialis* was sampled from Onagawa Bay, Miyagi, Japan in the spring of 2010. Both species were collected at the mature stage of gonadal development. Species discrimination of these two mussels was conducted by identification of adhesive protein sequence of each *Mytilus* species according to the method of Inoue et al. (1995). Only live, healthy shellfish were used for the experiments. All experimental procedures were completed promptly after opening the shell.

2.2. *Mytilus* ER cloning

Total RNA isolation and RACE PCR to isolate the full coding sequence of *Mytilus* ERs were performed as previously described (Treen et al., 2012). cDNA was synthesized from 1.5 μg of total RNA from the pedal ganglion and gonad. For *M. edulis* and *M. galloprovincialis*, cDNA was synthesized from the total RNA using Prime script Reverse Transcriptase (Takara, Tokyo, Japan) and the full transcripts were amplified by RACE PCR following the instructions of the SMART RACE cDNA synthesis kit (Takara). The following degenerate primers designed based on the DNA-binding domain and ligand-binding domain were used to find candidate genes of MeERs: MeER FW1 -5’ AAYGCNWSNGGNTTYCAYTAYGG 3’ and MeER RV -5’ TANCCNGGNACRTTYTTNGCCCA 3’ followed by nested PCR with MeER FW2 -5’ GCNWSNGGNTTYCAYTAYGGNGT 3’. The following gene specific primers were used: For MeER1 5’ RACE -5’ CACACGGTCTAAACGCACACCTT 3’, for MeER1 3’ RACE -5’

GCCAATCCTGACCCAGATCTTCAGGG 3' followed by nested PCR with 5' CCTGACCCAGATCTTCAGGGTGACGA 3', for MeER2 5' RACE -5' GGCCTGCAGACTGAGGAAGTGCTACG 3', for MeER2 3' RACE -5' CTGAGGAAGTGCTACGAAGTCGGCATG 3'. For *M. galloprovincialis* the cDNA sequences were amplified by RT-PCR with the following primers: For MgER1 - Forward primer: 5' ATGGAGCTTGATTTTACTGTG 3', reverse primer: 5' TCAGGAATCTGCCTCTAACATT 3', for MgER2 - forward primer 5' ATGGCCGAAAGGTTAAACTTTCCTG 3', reverse primer - 5' TCATGTTGGATTTTCCGTTTTG 3'. PCR products were subcloned into pGEM-T Easy vectors (Promega, WI, USA) and sequenced.

2.3. Sequence analysis

The open reading frames of the *Mytilus* cDNA sequences were translated and aligned with the sequences of ERs and ERRs taken from the NCBI database. The alignments were performed by CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustal2)(Chenna et al., 2003). A phylogenetic tree was constructed using the MEGA5 computer software (Tamura et al., 2011) using the ERs and ERRs from multiple species. The region from the start of the DNA binding domain to the end of the ligand-binding domain was used to generate the alignment and a phylogenetic tree was constructed using the maximum-likelihood method. A bootstrap test was calculated from 500 replicates.

2.4. Semi-quantitative RT-PCR for tissue distribution

cDNA was synthesized from total RNA extracted from the various organs (gill, adductor muscle, digestive gland, ovary, testis, spent gonad, and pedal ganglion) with the above-mentioned kit. RT-PCR was conducted with the gene specific primer sets: MeER1 Fw - 5' TTACGAGAAGGTGTGCGTTT 3', Rv - 5' TTTTTCACCATAGGAAGGATATGT 3'; MeER2 Fw - 5' GGAACACAAAGAAAAGAAAGGAAG 3', Rv - 5' ACAAATGTGTTCTGGATGGTG 3'; MeActin Fw - 5' AGAAAAGAGCTACGAATTGCC 3', Rv - 5' CTGTTGTATGTGGTTTCATGG 3'. For MeER1 and MeER2, the PCR conditions were 94^{o}C for 30 s, 50^{o}C for 30 s and 68^{o}C for 30 s for 25 cycles. For MeActin the PCR conditions were 94^{o}C for 30 s 57^{o}C for 30 s and 68^{o}C for 30 s for 20 cycles. PCR products were separated on 2.0%

196 agarose gels. An image of the gel was analyzed for the semi-quantitative RT-PCR
197 using Lane & Spot Analyzer software (ATTO, Tokyo, Japan). PCR products were
198 electrophoresed on 2% agarose gels.
199
200 2.5. *In situ* hybridization
201 *In situ* hybridization was performed on fixed gill and gonad tissue as previously
202 described (Itoh and Takahashi, 2007). In brief, single stranded RNA probes were
203 synthesized using DIG RNA labeling kit (Roche Diagnostics, Switzerland) according
204 to the manufacturer's instructions. The template for the probe was made by PCR of
205 *M. galloprovincialis* cDNA. The primers used for PCR amplification of MgER1 were:
206 forward primer - 5' TTACGAAAGGTGTGCGTTT 3', reverse primer - 5'
207 TTTTTCACCATAGGAAGGATATGT 3' and the primers for MgER2 were: forward
208 primer - 5' GGAACACAAAGAAAAGAAAGGAAG 3', reverse primer - 5'
209 ACAAATGTGTTCTGGATGGTG 3'. The PCR products corresponding to the D
210 (hinge) domain were subcloned into pGEM-T Easy vectors and the plasmid was
211 linearized by digestion with Spe I and Nco I. Probes for the sense and antisense DIG-
212 labeled RNA strands were transcribed *in vitro* from the linearized cDNA plasmid. The
213 sections were hybridized with the DIG labeled probes overnight at 45^{o}C and
214 visualized with NBT/BCIP (Roche Diagnostics, USA).
215
216 2.6. Semi-quantitative RT-PCR for the cultured ovaries with E_2 or T
217 Approximately 20 mg of *M. edulis* ovary tissue was dissected and then cultured for 2
218 days at 10^{o}C. The methods for tissue culture and condition of media were the same as
219 previously described (Nakamura et al., 2007). Tissue was treated with either estradiol-
220 17ß (E_2) or testosterone (T) at 10^{-6} M concentration, which is dissolved with dimethyl
221 sulfoxide (DMSO) and diluted with the modified Herbst's artificial seawater (ASW).
222 The control group contained 0.01% DMSO alone. After the short-time culture, total
223 RNA was isolated and cDNA was synthesized as described above. Four experimental
224 replicates were set per treatment group and sampled for further RNA extraction.
225 Changes of gene expression for the MeER1, MeER2 and MeActin was analyzed by
226 semi-quantitative RT-PCR with above mentioned GSP sets and PCR conditions. For
227 selection of suitable reference gene for the data normalization, MeActin was chosen
228 as a stable reference gene which has already validated in the previous qPCR report of
229 *M. edulis* reproductive tissues treated with E_2-exposure (Cubero-Leon et al., 2012).

2.7. Data analysis

Gene expression differences among ovarian tissues cultured with E_2, T, and ASW were tested with ANOVA followed by Turkey's test ($p < 0.05$).

3. Results

3.1. Cloning and protein domain prediction of *Mytilus* estrogen receptor transcripts

We successfully cloned the full-length coding cDNA sequences of 4 estrogen receptors: 2 from *M. edulis* and 2 from *M. galloprovincialis*. The cDNA sequences were used to predict the open reading frames, these sequences indicated a strong similarity to known estrogen receptor sequences. The genes that these transcripts encode have tentatively been named MeER1 and MeER2 for the *M. edulis* sequences, and MgER1 and MgER2 for the *M. galloprovincialis* sequences. MeER1 and MgER1 have identical amino acid sequences, but slightly different mRNA sequences, as MeER2 and MgER2 do. These sequences have been submitted to Genbank (Accession Nos: MeER1 - **BAF34365**, MeER2 - **BAF34366**, MgER1 - **BAJ07193**, MgER2 - **BAF34908**). The *Mytilus* ERs were compared with the ERs from *Capitella capitata* and *Marisa cornuarietis* as well as the ERRs form *M. cornuarietis* and *Drosophila melanogaster* (Fig. 1a). The alignment was made with only the highly conserved C, D and E domains corresponding to the DNA binding domains, the hinge region connecting C to E domains and the ligand-binding domain, respectively. The alignments showed that Me/MgER2 was similar to the estrogen receptors and Me/MgER1 was similar to the ERRs. The C domain (DNA binding domains; DBD) showed a very high level of conservation for all sequences. The E domain (ligand-binding domain; LBD) that constitutes the ligand-binding pocket was less well conserved (Fig. 1a). The detailed comparison of LBDs indicates that the conserved residues for ligand binding in Me/MgER2 were especially found in the helix 7 of human ER alpha (Supplementary figure 1), whereas Me/MgER1 displayed no conserved residue. In addition, the blastp comparison of LBDs found that Me/Mg ER2 showed the highest identity number (36%) to human ER alpha among Lophotrochozoa selected (Supplementary table 1). The schematic representation of

both Me/MgER1 and Me/MgER2 based on their mRNA sequences showed a typical organization with distinct A-E domains and 5'- and 3'-untranslated regions (Fig. 1b).

3.2. Phylogenetic analysis of ERs and ERRs

Using the 4 full-length coding *Mytilus* ER sequences obtained in this study, we performed a phylogenetic analysis with several ERs and ERRs (Fig. 2). Concentrating mainly on mollusk sequences, but with some mammalian, annelid and insect sequences to provide a clearer evolutionary context, MeER2 and MgER2, as well as MeER1 and MgER1 are most similar to each other. Since these species are very closely related and the protein sequences are almost identical. Our analysis showed 2 distinct groups corresponding to ERs and ERRs. The Me/MgER2 sequences were in the ER group and the Me/MgER1 sequences were in the ERR group. Among mollusk ER sequences, the *Mytilus* ER sequences are closest to the other bivalve (oyster) ER. The gastropod ERs form a cluster, and the cephalopod *Octopus* ER is distinct from the bivalve and gastropod ER clusters. The human and annelid ERs formed outgroups. Interestingly the annelid ER appeared more divergent from the mollusk species than the mammalian ERs. The *Mytilus* ER1 sequences were most similar to the *Marisa* ERR sequence. These 3 mollusk ERRs were grouped together with the human and insect ERRs.

3.3. Tissue distribution of ER1 and ER2 transcripts

Tissue distribution of both ER transcripts was analyzed in various *Mytilus* (*M. edulis*) tissues. In brief, both ER transcripts were broadly expressed in all tissues examined (Fig. 3). In particular, relatively high level of ER1 transcripts were detected in gill, digestive gland, whereas ER2 transcripts were abundantly detected in ovary and pedal ganglion.

3.4. Visualization of ER1 and ER2 transcripts in ovary, testis, and gill

The mRNA expression of ER1 and ER2 in the gill, testis and ovary of *Mytilus* (*M. galloprovincialis*) was visualized by *in situ* hybridization (Fig. 4). High levels of expression could be seen in all tissues tested for both ER forms. Gill tissue appeared to have an especially high level of expression. In the ovary, the vitellogenic oocytes and follicle cells in contact with the oocyte showed the positive signal for both ER mRNAs. Both ER mRNAs were expressed in the Sertoli cells along the inside of the

297 acinar wall in the testis. In rare cases, faint background was seen in control slides
298 hybridized with sense probes (Fig. 4 insets), but this signal was negligibly weak in
299 comparison with the signals obtained in all slides with anti-sense probes.
300
301 3.5. Transcriptional response of ER and ERR in organ-cultured *Mytilus* ovary exposed
302 to vertebrate-type steroids
303 *Mytilus* (*M. edulis*) ovarian tissue was *in vitro*-cultured with either T or E_2, and then
304 the transcriptional response of ER and ERR was assessed (Fig. 5). Incubations with
305 both steroids for 2 days did not significantly change the expression levels for MeER1,
306 whereas MeER2 expression was upregulated by exposure to both steroids.
307 Particularly, E_2 induced a significant increase of expression level approximately
308 double that of the control.
309
310
311 **4. Discussion**
312
313 We successfully cloned the full-length coding sequences of two forms of ERs from
314 two *Mytilus* bivalves: *M. edulis* and *M. galloprovincialis*. One form showed similarity
315 to known ERs and the other form showed similarity to ERRs. These two SRs were
316 shown to be present in both *Mytilus* species. The multiple alignment analysis found
317 that the DBD shows a very high level of conservation, whereas the LBD shows a less
318 conservation among ERs and ERRs. Specifically in the LBD, Me/MgER2 possess
319 many conserved residues for ligand binding in human ER alpha (Tanenbaum et al.,
320 1998) and relatively high sequence similarity rather than Me/MgER1 and other
321 Lophotrochozoa, suggesting its possible interaction to E_2. The protein domain
322 prediction identified that both *Mytilus* ERs and ERRs have the "A-E" domains that
323 are commonly seen in other species. Our phylogenetic analysis of multiple
324 protostome and deuterostome ERs and ERRs shows clear separation into two groups.
325 Most of known mollusk SR sequences are ERs constituting the ER branch of the tree,
326 but a few of mollusk ERRs identified in *Marisa* and *Mytilus* (this study) are separated
327 into the ERR branch. Our cloning results of *Mytilus* ERs and ERRs indicate that two
328 different species in bivalve possess at least an ER and an ERR as known in the
329 gastropod (Bannister et al., 2007). Therefore, it is probable that possessing an ER and
330 an ERR is a fundamental system for most mollusks and that an ERR might exist for

331 the species like *Aplysia californica* and *Crassostrea gigas* where currently only a
332 single ER form is known.

333 Our results individually detecting ER and ERR transcripts in *Mytilus* indicate
334 that both transcripts are abundantly expressed in multiple locations including gonads
335 and gills. The presence of both transcripts in the gonads would immediately suggest a
336 role in bivalve reproduction (Croll and Wang, 2007). In fact, both MgER1 and
337 MgER2 transcripts were localized in the follicle cells in female and Sertoli cells in
338 males through which nutrients are channeled into the developing gametes (Pipe,
339 1987). The presence in the gills is interesting since these organs are constantly
340 exposed to the seawater in order to provide a stream of fresh seawater for respiration
341 as well as to filter for food particles. In terms of endocrine disruption model by
342 industrial pollutants (Jobling et al., 2004; Ciocan et al., 2010), the molecular behavior
343 of ERs in gills may be a worth analyzing for assessing the local effect of EDCs in the
344 peripheral tissues which might minimize the possibility of interference by endogenous
345 vertebrate-like estrogen found in *Mytilus* (Reis-Henriques et al., 1990; Zhu et al.,
346 2003).

347 Our *in vitro* culture experiment showed that the presence of E_2 (10^{-6} M
348 concentration) in culture media is capable of upregulating the expression of MeER2
349 (ER) and its expression was also upregulated by T, which might be converted to
350 estrogens partially. Whereas no significant changes were seen in MeER1 (ERR)
351 indicating no response to neither E_2 or T, even though the concentrations were
352 relatively high compared to our previous *in vivo* experiments (Puinean et al., 2006;
353 Ciocan et al., 2010). In addition to the effect of E_2, the previous *in vivo* study reported
354 that a significant increase in MeER2 expression was observed the gonads in *M. edulis*
355 exposed to the E_2 (exposed to 5 - 200 ng/L) or synthetic estrogens (i.e. ethinyl
356 estrogen, EE_2 (exposed to 5 – 50 ng/L); estradiol benzoate, EB (exposed 200 ng/L)) at
357 the early stage of gametogenesis (Ciocan et al., 2010), assuming that E_2 might act for
358 MeER2 as a functional ligand. This E_2-dependent alteration for elevating ER
359 transcript level has already been confirmed in teleost species by showing that the
360 levels of three goldfish ER subtypes were differentially regulated by E_2 level (Marlatt
361 et al., 2008). Therefore, this autonomous regulation mechanism of ER transcription
362 may be present in *M. edulis* resulting in the increase of sensitivity to the possible
363 ligands. Further studies might be able to examine other vertebrate-type estrogens such
364 as estrone (E_1) and estriol (E_3) with E_2 at lower doses (e.g. 10^{-9} M and much lower) in

365 order to clarify whether this autonomous regulation is an E_2-specific response and
366 dose dependent. However this experiment could be difficult since their sensitivity can
367 be also affected by the endogenous estrogen-like steroid level that varies in
368 association with reproductive stages reported in scallop (Osada et al., 2004).
369 In addition to E_2-dependent disruption, the environmental exposure of *Mytilus*
370 to estrogens was not observed to change expression of vitellogenin (Puinean et al.,
371 2006), but could disrupt the expression of serotonin receptors (5-HT receptor) and
372 cyclooxygenase (COX) (Cubero-Leon et al., 2010). Nevertheless this variability of
373 MeER2 and other gene expressions in *Mytilus* induced by vertebrate-type estrogens,
374 their ligand specificity and affinity to MeER2 need to be further confirmed since this
375 study did not investigate this. By the sequential investigations from Thornton' group,
376 reporter or binding assays with vertebrate-type estrogens have accumulated evidence
377 that the mollusk ERs are constitutively active transcription receptors, meaning that the
378 mollusk ERs are ligand-independently regulated transcription factors (Bridgham et al.,
379 2014) but that annelid ERs are only activated by the vertebrate-type estrogen (E_2) in
380 the Superphylum Lophotrochozoa (Keay and Thornton, 2009). According to their
381 findings, *Mytilus* ERs might also behave as a ligand-independent transcription factors.
382 If so, the elevation of MeER2 expression with E_2 in this study would be explained by
383 the non-genomic response mechanism to estrogens (Canesi et al., 2004a; Canesi et al.,
384 2004b). They previously proposed the possible presence of an indirect pathway of E_2
385 signaling in *Mytilus* hemocytes which can moderate several kinase-mediated cascades
386 by changing phosphorylation state of transcription factors, also it was shown that ERs
387 are likely to exist in these hemocytes (Canesi et al., 2004a). Similar disruptive effects
388 were seen to occur in both *in vitro* and *in vivo* assays (Canesi et al., 2007b), as well as
389 in the digestive gland (Canesi et al., 2007a). These results in *Mytilus* could involve the
390 putative indirect auto-regulation mechanism of MeER2 through a non-genomic
391 pathway in gonadal cells as well.
392 In conclusion, this study first reports the molecular identification of full-length
393 coding sequence of an ER and an ERR in two *Mytilus* species. Our phylogenetic
394 analysis clearly visualize the genetic evolution of ERs and ERRs of *Mytilus* species.
395 The detection of ER and ERR transcripts in the *Mytilus* gonads with E_2-dependent
396 variability identified the different molecular behavior in response to vertebrate-type
397 steroids between ER and ERR, implicating the essential mechanism of ER in *Mytilus*

reproduction and that their gametogenesis are potentially disrupted by hormone-disrupting substances including vertebrate-type steroids.

Acknowledgements

We warmly thank Mr. Peter Darnell, Indian Point Mussel Farm, Nova Scotia, Canada, for courtesy of mussels. This work was supported by JSPS KAKENHI (23380109), Joint Project of Japan-UK Research Cooperative Program from JSPS and "Tohoku Ecosystem-Associated Marine Science (TEAMS)" research project funds from MEXT (to M.O.).

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Figure legends

Fig. 1 A comparison of protostome estrogen receptors.
(A) CLUSTALW alignment of amino acid sequences of estrogen receptors (ER/ER2) and estrogen related receptors (ERR/ER1). DBD; DNA-binding domain, LBD; ligand-binding domain. Amino acids conserved between species for a single type of receptor are colored in yellow or blue. Amino acids conserved between both types of receptor are colored in green. (B) Illustration of the MeER1 and MeER2 cDNA transcripts. The boxed region indicates regions that are translated to proteins. Letters A-E indicate the relative locations of the estrogen receptor domains. Species names and Genbank accession numbers: *Mytilus edulis*: MeER1 (**BAF34365**), MeER2 (**BAF34366**), *Mytilus galloprovincialis*: MgER1 (**BAJ07193**), MgER2 (**BAF34908**), *Capitella capitata*: *Capitella* ER (**ACD11039**), *Marisa cornuarietis*: *Marisa* ER (**ABI97117**), *Marisa* ERR (**ABI97120**), *Drosophila melanogaster*: *Drosophila* ERR (**NP648183**).

Fig. 2 Phylogenetic tree generated from the alignment of multiple estrogen receptors and estrogen related receptors. Sequences first described in this study are indicated by an asterisk (*). The human androgen receptor (Human AR) is included as an outgroup. Bootstrap values are indicated at nodes. Scale bare indicates an expected changes per site. Species names and Genbank accession numbers: *Thais clavigera*: *Thais* ER (**BAC66480**), *Nucella lapillus*: *Nucella* ER (**ABQ96884**), *Marisa cornuarietis*: *Marisa* ER (**ABI97117**), *Marisa* ERR (**ABI97120**), *Aplysia californica*: *Aplysia* ER (**NP_001191648**), *Octopus vulgaris*: *Octopus* ER (**ABG00286**), *Mytilus edulis*: MeER1 (**BAF34365**), MeER2 (**BAF34366**), *Mytilus galloprovincialis*: MgER1 (**BAJ07193**), MgER2 (**BAF34908**), *Crassostrea gigas*: Oyster ER (**BAF45381**), *Homo sapiens*: Human ER-alpha (**CAA27284**), Human ER-beta (**CAA67555**), Human ERR-alpha (**NP_004442**), Human ERR-beta (**NP_004443**), Human ERR-gamma (**NP996317**), Human AR (**ADK91081**), *Capitella capitata*: *Capitella* ER (**ACD11039**), *Drosophila melanogaster*: *Drosophila* ERR (**NP648183**).

Fig. 3 Representative tissue distribution of estrogen receptor transcripts in *Mytilus*. Black and gray bars show relative mRNA expression of MeER1 (A) and MeER2 (B), respectively in various tissues (gill, AM; adductor muscle, DG; digestive

gland, OV, ovary, TES; testis, SPENT; spent gonad, PG; pedal ganglion). Actin was used as an endogenous reference gene.

Fig. 4 *In situ* hybridization of estrogen receptor transcripts in *Mytilus* gonads and gills. Tissue type is indicated by rows and different probes are indicated by the columns. Sense strand hybridizations are shown as insets. OC; oocyte, FC; follicle cell, SC; Sertoli cell, CC; ciliated cell. Scale bars = 50 µm

Fig. 5 *In vitro* effects of estradiol-17ß and testosterone on estrogen receptor transcription in the *Mytilus* ovarian tissue cultured for two days. Dark and light gray bars show relative mRNA expression of MeER1 (A) and MeER2 (B) mRNA expression (n = 4 per treatment group, means ± S.E.). Tissue was treated with either estradiol-17ß (E_2) or testosterone (T) at 10^{-6} M concentration. An asterisk (*) indicates values that are significantly different from control ($p < 0.05$). E; estradiol-17ß, T; testosterone

Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two *Mytilus* species.

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Running title:
Molecular characterization of *Mytilus* estrogen receptors

This ms has 17 pages, 5 figures

Abstract

Vertebrate-like sex steroid hormones have been widely detected in mollusks, and numerous experiments have shown the importance of steroids in gonad development. Nevertheless, their signaling pathways in invertebrates have not been uncovered yet. Steroid receptors are an ancient class of transcription factors with multiple roles in not only vertebrates but also invertebrates. Estrogen signaling is thought to have major roles in mollusk physiology, but the full repertoire of estrogen receptors is unknown. We presented the successful cloning of two novel forms of estrogen receptor-like genes. These receptors are present in two closely related species of *Mytilus*: *Mytilus edulis* and *Mytilus galloprovincialis*, commonly known and widely distributed sentinel species. Our phylogenetic analysis revealed that one of these receptors is an estrogen receptor (ER) and the other one is an estrogen-related receptor (ERR). Studies of expression analysis showed that both receptor mRNAs were localized in the oocytes and follicle cells in contact with developing oocytes in the ovary and Sertoli cells in the testis, and in the ciliated cells of the gill. In addition, we have evidence that one (ER) of these may have a capacity to autoregulate its own expression in the gonadal cells by estrogen (E_2) and that this gene is responsive to estrogenic compounds.

Highlights:

- We cloned two forms of full-length ER-like genes from two *Mytilus* species.
- One is an ER and the other one is an ERR.
- Possessing an ER and an ERR may be a fundamental system for most mollusks.
- Their mRNAs were both detected in the oocytes, follicle cells, and Sertoli cells.
- ER transcription may be autonomously upregulated in the gonadal cells by E_2 treatment.

Key words: mussel, reproduction, endocrine disruption, autoregulation

1. Introduction

Many of controversial topics still persist on the knowledge of invertebrate steroid hormones with their receptors even though numerous studies have been conducted for more than five decades (Hultin et al., 2014). These arguments can be attributed to the unresolved issue that there has never been concrete evidence for the presence of endogenous sex steroid hormones in invertebrates despite evidence indicating the possible presence of invertebrate estradiol-17β (E_2) (Reis-Henriques et al., 1990; Zhu et al., 2003). In mollusks, Scott (2012; 2013) concerned the interference of a non-specific assay for detecting mollusk steroids based on the detection method for vertebrate-type steroids because no conclusive evidence exists for endogenous steroid hormones in any mollusk. Nevertheless vertebrate-type steroids that respond along with their reproductive cycle or exposure to endocrine disrupting chemicals (EDCs) (Scott, 2013) have been detected in many mollusk species (Janer and Porte, 2007; Matsumoto et al., 2007). Janer and Porte (2007) systematically summarized the possible sex steroidogenic pathways in several invertebrates (i.e. echinoderms, crustaceans, and mollusks). In the recent review, Giusti and Joaquim-Justo (2013) proposed that the endogenous pathway of steroid hormone synthesis (i.e. esterification) and biologically-active vertebrate-like steroid hormones exist in at least three mollusk classes: Bivalvia, Gastropoda, and Cephalopoda. Nevertheless, there has been no firm evidence of the presence for functional receptors to vertebrate-like steroids and that vertebrate-like steroids function on mollusk endocrine systems via their specific receptors (Scott, 2012).

Intriguingly, Thornton's lab has investigated the evolutionary divergence in physiological activity of steroid hormone receptors (SR) especially for lophotrochozoan ERs with their ligand-specific activity (Thornton et al., 2003; Keay and Thornton, 2009; Eick and Thornton, 2011). A recent study combining evolutional genetics and protein structure from his group delivered an interesting insight in lophotrochozoan SR biology including ERs (Bridgham et al., 2014). They reported that annelid ERs are activated by the vertebrate-type estrogen (E_2) (Keay and Thornton, 2009), whereas several other mollusk ERs (e.g. Aplysia (Thornton et al., 2003), land snail, limpet, marine snail (Kajiwara et al., 2006), octopus (Keay et al., 2006) and oyster (Matsumoto et al., 2007)) are constitutively active transcription receptors. Using X-ray crystallography to investigate the structure, they also found a

clue to the regulatory mechanism in the constitutive transcriptional activity of ER by showing that structural variation of the ligand-binding pocket filled with bulky residues preventing ligand occupancy in oyster ER (Bridgham et al., 2014).

In terms of molecular evolution of ER, vertebrates are known to have multiple ERs and estrogen-related receptors (ERRs) as part of their SR repertoire. Estrogen receptors (ERs) are a group of nuclear receptor superfamily of steroid hormones classified as ligand-activated transcription factors that modulate gene expression. Whereas ERRs are orphan nuclear receptors possessing similar structure and sequence similarity to ERs. However, ERRs bind to not only estrogen response elements but also their own response elements (ERR response elements), and then modulate transcription but are not activated by estrogens (Horard and Vanacker, 2003). Both ERs and ERRs are widely distributed in different tissue types, however there are some notable differences in their expression patterns (Bookout et al., 2006). The ERs and ERRs are found in diverse species throughout the animal kingdom. Evolutionary genetics has revealed that two distinct subtypes of nuclear ERs (ERα and ERβ) act in vertebrates including mammals (Keaveney et al., 1991) and single ER is currently identified in a wide variety of invertebrates such as nematodes (Mimoto et al., 2007), annelids and mollusks (Eick and Thornton, 2011). Whereas, the mammals possesses three different subtypes of ERR (ERR-α, ERR-β, and ERR-γ) in their genome (Horard and Vanacker, 2003). In invertebrates, the ERRs are not very well understood. Baker (2008) reported that the sequenced *Trichoplax* genome has an ERR, but no ER. Furthermore, additional BLAST searches found ERRs in other three lophotrochozoan transcriptomes: an annelid worm, a leech, and a snail. Whereas invertebrate-like ERs exist in worm and snail, but not in leech. Another report showed that the freshwater snail *Marisa cornuarietis* has two transcripts that encode an invertebrate-like ER and an ERR (Bannister et al., 2007). However, there has not been enough examples which verify the presence of both invertebrate-like ER and an ERR in mollusks.

In the present study, we focused on two *Mytilus* species: *Mytilus edulis* and *Mytilus galloprovincialis* as commonly known and widely distributed sentinel species which are potentially susceptible to EDCs. We previously reported that a significant increase of *vitellogenin* (VTG) and ER2 transcripts was caused by estrogen exposure in *M. edulis* at the early stage of gametogenesis, but not at mature stages (Puinean et al., 2006; Ciocan et al., 2010). Nevertheless in these *in vivo* studies, invertebrate-like

ER and ERR have not been fully investigated in *Mytilus* species yet from the point of view of evolutionary genetics and molecular characterization. To build the basic knowledge of invertebrate-like ER in bivalves, this study aimed to clone the full-length coding cDNA sequence of both invertebrate-like ER and ERR from two *Mytilus* species, and to characterize their molecular behavior, particularly gene expression responses to estrogen exposure.

2. Materials and methods

2.1. Animals

Mytilus edulis was sampled from Brighton, UK and provided from Indian Point Mussel Farm, Canada in the summer of 2010, and *Mytilus galloprovincialis* was sampled from Onagawa Bay, Miyagi, Japan in the spring of 2010. Both species were collected at the mature stage of gonadal development. Species discrimination of these two mussels was conducted by identification of adhesive protein sequence of each *Mytilus* species according to the method of Inoue et al. (1995). Only live, healthy shellfish were used for the experiments. All experimental procedures were completed promptly after opening the shell.

2.2. *Mytilus* ER cloning

Total RNA isolation and RACE PCR to isolate the full coding sequence of *Mytilus* ERs were performed as previously described (Treen et al., 2012). cDNA was synthesized from 1.5 μg of total RNA from the pedal ganglion and gonad. For *M. edulis* and *M. galloprovincialis*, cDNA was synthesized from the total RNA using Prime script Reverse Transcriptase (Takara, Tokyo, Japan) and the full transcripts were amplified by RACE PCR following the instructions of the SMART RACE cDNA synthesis kit (Takara). The following degenerate primers designed based on the DNA-binding domain and ligand-binding domain were used to find candidate genes of MeERs: MeER FW1 -5’ AAYGCNWSNGGNTTYCAYTAYGG 3’ and MeER RV -5’ TANCCNGGNACRTTYTTNGCCCA 3’ followed by nested PCR with MeER FW2 -5’ GCNWSNGGNTTYCAYTAYGGNGT 3’. The following gene specific primers were used: For MeER1 5’ RACE -5’ CACACGGTCTAAACGCACACCTT 3’, for MeER1 3’ RACE -5’

GCCAATCCTGACCCAGATCTTCAGGG 3' followed by nested PCR with 5' CCTGACCCAGATCTTCAGGGTGACGA 3', for MeER2 5' RACE -5' GGCCTGCAGACTGAGGAAGTGCTACG 3', for MeER2 3' RACE -5' CTGAGGAAGTGCTACGAAGTCGGCATG 3'. For *M. galloprovincialis* the cDNA sequences were amplified by RT-PCR with the following primers: For MgER1 - Forward primer: 5' ATGGAGCTTGATTTTACTGTG 3', reverse primer: 5' TCAGGAATCTGCCTCTAACATT 3', for MgER2 - forward primer 5' ATGGCCGAAAGGTTAAACTTTCCTG 3', reverse primer - 5' TCATGTTGGATTTTCCGTTTTG 3'. PCR products were subcloned into pGEM-T Easy vectors (Promega, WI, USA) and sequenced.

2.3. Sequence analysis

The open reading frames of the *Mytilus* cDNA sequences were translated and aligned with the sequences of ERs and ERRs taken from the NCBI database. The alignments were performed by CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustal2)(Chenna et al., 2003). A phylogenetic tree was constructed using the MEGA5 computer software (Tamura et al., 2011) using the ERs and ERRs from multiple species. The region from the start of the DNA binding domain to the end of the ligand-binding domain was used to generate the alignment and a phylogenetic tree was constructed using the maximum-likelihood method. A bootstrap test was calculated from 500 replicates.

2.4. Semi-quantitative RT-PCR for tissue distribution

cDNA was synthesized from total RNA extracted from the various organs (gill, adductor muscle, digestive gland, ovary, testis, spent gonad, and pedal ganglion) with the above-mentioned kit. RT-PCR was conducted with the gene specific primer sets: MeER1 Fw - 5' TTACGAGAAGGTGTGCGTTT 3', Rv - 5' TTTTTCACCATAGGAAGGATATGT 3'; MeER2 Fw - 5' GGAACACAAAGAAAAGAAAGGAAG 3', Rv - 5' ACAAATGTGTTCTGGATGGTG 3'; MeActin Fw - 5' AGAAAAGAGCTACGAATTGCC 3', Rv - 5' CTGTTGTATGTGGTTTCATGG 3'. For MeER1 and MeER2, the PCR conditions were 94^{o}C for 30 s, 50^{o}C for 30 s and 68^{o}C for 30 s for 25 cycles. For MeActin the PCR conditions were 94^{o}C for 30 s 57^{o}C for 30 s and 68^{o}C for 30 s for 20 cycles. PCR products were separated on 2.0%

196 agarose gels. An image of the gel was analyzed for the semi-quantitative RT-PCR
197 using Lane & Spot Analyzer software (ATTO, Tokyo, Japan). PCR products were
198 electrophoresed on 2% agarose gels.
199
200 2.5. *In situ* hybridization
201 *In situ* hybridization was performed on fixed gill and gonad tissue as previously
202 described (Itoh and Takahashi, 2007). In brief, single stranded RNA probes were
203 synthesized using DIG RNA labeling kit (Roche Diagnostics, Switzerland) according
204 to the manufacturer's instructions. The template for the probe was made by PCR of
205 *M. galloprovincialis* cDNA. The primers used for PCR amplification of MgER1 were:
206 forward primer - 5' TTACGAAAGGTGTGCGTTT 3', reverse primer - 5'
207 TTTTTCACCATAGGAAGGATATGT 3' and the primers for MgER2 were: forward
208 primer - 5' GGAACACAAAGAAAAGAAAGGAAG 3', reverse primer - 5'
209 ACAAATGTGTTCTGGATGGTG 3'. The PCR products corresponding to the D
210 (hinge) domain were subcloned into pGEM-T Easy vectors and the plasmid was
211 linearized by digestion with Spe I and Nco I. Probes for the sense and antisense DIG-
212 labeled RNA strands were transcribed *in vitro* from the linearized cDNA plasmid. The
213 sections were hybridized with the DIG labeled probes overnight at 45^{o}C and
214 visualized with NBT/BCIP (Roche Diagnostics, USA).
215
216 2.6. Semi-quantitative RT-PCR for the cultured ovaries with E_2 or T
217 Approximately 20 mg of *M. edulis* ovary tissue was dissected and then cultured for 2
218 days at 10^{o}C. The methods for tissue culture and condition of media were the same as
219 previously described (Nakamura et al., 2007). Tissue was treated with either estradiol-
220 17ß (E_2) or testosterone (T) at 10^{-6} M concentration, which is dissolved with dimethyl
221 sulfoxide (DMSO) and diluted with the modified Herbst's artificial seawater (ASW).
222 The control group contained 0.01% DMSO alone. After the short-time culture, total
223 RNA was isolated and cDNA was synthesized as described above. Four experimental
224 replicates were set per treatment group and sampled for further RNA extraction.
225 Changes of gene expression for the MeER1, MeER2 and MeActin was analyzed by
226 semi-quantitative RT-PCR with above mentioned GSP sets and PCR conditions. For
227 selection of suitable reference gene for the data normalization, MeActin was chosen
228 as a stable reference gene which has already validated in the previous qPCR report of
229 *M. edulis* reproductive tissues treated with E_2-exposure (Cubero-Leon et al., 2012).

2.7. Data analysis

Gene expression differences among ovarian tissues cultured with E_2, T, and ASW were tested with ANOVA followed by Turkey's test ($p < 0.05$).

3. Results

3.1. Cloning and protein domain prediction of *Mytilus* estrogen receptor transcripts

We successfully cloned the full-length coding cDNA sequences of 4 estrogen receptors: 2 from *M. edulis* and 2 from *M. galloprovincialis*. The cDNA sequences were used to predict the open reading frames, these sequences indicated a strong similarity to known estrogen receptor sequences. The genes that these transcripts encode have tentatively been named MeER1 and MeER2 for the *M. edulis* sequences, and MgER1 and MgER2 for the *M. galloprovincialis* sequences. MeER1 and MgER1 have identical amino acid sequences, but slightly different mRNA sequences, as MeER2 and MgER2 do. These sequences have been submitted to Genbank (Accession Nos: MeER1 - **BAF34365**, MeER2 - **BAF34366**, MgER1 - **BAJ07193**, MgER2 - **BAF34908**). The *Mytilus* ERs were compared with the ERs from *Capitella capitata* and *Marisa cornuarietis* as well as the ERRs form *M. cornuarietis* and *Drosophila melanogaster* (Fig. 1a). The alignment was made with only the highly conserved C, D and E domains corresponding to the DNA binding domains, the hinge region connecting C to E domains and the ligand-binding domain, respectively. The alignments showed that Me/MgER2 was similar to the estrogen receptors and Me/MgER1 was similar to the ERRs. The C domain (DNA binding domains; DBD) showed a very high level of conservation for all sequences. The E domain (ligand-binding domain; LBD) that constitutes the ligand-binding pocket was less well conserved (Fig. 1a). The detailed comparison of LBDs indicates that the conserved residues for ligand binding in Me/MgER2 were especially found in the helix 7 of human ER alpha (Supplementary figure 1), whereas Me/MgER1 displayed no conserved residue. In addition, the blastp comparison of LBDs found that Me/Mg ER2 showed the highest identity number (36%) to human ER alpha among Lophotrochozoa selected (Supplementary table 1). The schematic representation of

both Me/MgER1 and Me/MgER2 based on their mRNA sequences showed a typical organization with distinct A-E domains and 5'- and 3'-untranslated regions (Fig. 1b).

3.2. Phylogenetic analysis of ERs and ERRs

Using the 4 full-length coding *Mytilus* ER sequences obtained in this study, we performed a phylogenetic analysis with several ERs and ERRs (Fig. 2). Concentrating mainly on mollusk sequences, but with some mammalian, annelid and insect sequences to provide a clearer evolutionary context, MeER2 and MgER2, as well as MeER1 and MgER1 are most similar to each other. Since these species are very closely related and the protein sequences are almost identical. Our analysis showed 2 distinct groups corresponding to ERs and ERRs. The Me/MgER2 sequences were in the ER group and the Me/MgER1 sequences were in the ERR group. Among mollusk ER sequences, the *Mytilus* ER sequences are closest to the other bivalve (oyster) ER. The gastropod ERs form a cluster, and the cephalopod *Octopus* ER is distinct from the bivalve and gastropod ER clusters. The human and annelid ERs formed outgroups. Interestingly the annelid ER appeared more divergent from the mollusk species than the mammalian ERs. The *Mytilus* ER1 sequences were most similar to the *Marisa* ERR sequence. These 3 mollusk ERRs were grouped together with the human and insect ERRs.

3.3. Tissue distribution of ER1 and ER2 transcripts

Tissue distribution of both ER transcripts was analyzed in various *Mytilus* (*M. edulis*) tissues. In brief, both ER transcripts were broadly expressed in all tissues examined (Fig. 3). In particular, relatively high level of ER1 transcripts were detected in gill, digestive gland, whereas ER2 transcripts were abundantly detected in ovary and pedal ganglion.

3.4. Visualization of ER1 and ER2 transcripts in ovary, testis, and gill

The mRNA expression of ER1 and ER2 in the gill, testis and ovary of *Mytilus* (*M. galloprovincialis*) was visualized by *in situ* hybridization (Fig. 4). High levels of expression could be seen in all tissues tested for both ER forms. Gill tissue appeared to have an especially high level of expression. In the ovary, the vitellogenic oocytes and follicle cells in contact with the oocyte showed the positive signal for both ER mRNAs. Both ER mRNAs were expressed in the Sertoli cells along the inside of the

297 acinar wall in the testis. In rare cases, faint background was seen in control slides
298 hybridized with sense probes (Fig. 4 insets), but this signal was negligibly weak in
299 comparison with the signals obtained in all slides with anti-sense probes.
300
301 3.5. Transcriptional response of ER and ERR in organ-cultured *Mytilus* ovary exposed
302 to vertebrate-type steroids
303 *Mytilus* (*M. edulis*) ovarian tissue was *in vitro*-cultured with either T or E_2, and then
304 the transcriptional response of ER and ERR was assessed (Fig. 5). Incubations with
305 both steroids for 2 days did not significantly change the expression levels for MeER1,
306 whereas MeER2 expression was upregulated by exposure to both steroids.
307 Particularly, E_2 induced a significant increase of expression level approximately
308 double that of the control.
309
310
311 **4. Discussion**
312
313 We successfully cloned the full-length coding sequences of two forms of ERs from
314 two *Mytilus* bivalves: *M. edulis* and *M. galloprovincialis*. One form showed similarity
315 to known ERs and the other form showed similarity to ERRs. These two SRs were
316 shown to be present in both *Mytilus* species. The multiple alignment analysis found
317 that the DBD shows a very high level of conservation, whereas the LBD shows a less
318 conservation among ERs and ERRs. Specifically in the LBD, Me/MgER2 possess
319 many conserved residues for ligand binding in human ER alpha (Tanenbaum et al.,
320 1998) and relatively high sequence similarity rather than Me/MgER1 and other
321 Lophotrochozoa, suggesting its possible interaction to E_2. The protein domain
322 prediction identified that both *Mytilus* ERs and ERRs have the "A-E" domains that
323 are commonly seen in other species. Our phylogenetic analysis of multiple
324 protostome and deuterostome ERs and ERRs shows clear separation into two groups.
325 Most of known mollusk SR sequences are ERs constituting the ER branch of the tree,
326 but a few of mollusk ERRs identified in *Marisa* and *Mytilus* (this study) are separated
327 into the ERR branch. Our cloning results of *Mytilus* ERs and ERRs indicate that two
328 different species in bivalve possess at least an ER and an ERR as known in the
329 gastropod (Bannister et al., 2007). Therefore, it is probable that possessing an ER and
330 an ERR is a fundamental system for most mollusks and that an ERR might exist for

331 the species like *Aplysia californica* and *Crassostrea gigas* where currently only a
332 single ER form is known.

333 Our results individually detecting ER and ERR transcripts in *Mytilus* indicate
334 that both transcripts are abundantly expressed in multiple locations including gonads
335 and gills. The presence of both transcripts in the gonads would immediately suggest a
336 role in bivalve reproduction (Croll and Wang, 2007). In fact, both MgER1 and
337 MgER2 transcripts were localized in the follicle cells in female and Sertoli cells in
338 males through which nutrients are channeled into the developing gametes (Pipe,
339 1987). The presence in the gills is interesting since these organs are constantly
340 exposed to the seawater in order to provide a stream of fresh seawater for respiration
341 as well as to filter for food particles. In terms of endocrine disruption model by
342 industrial pollutants (Jobling et al., 2004; Ciocan et al., 2010), the molecular behavior
343 of ERs in gills may be a worth analyzing for assessing the local effect of EDCs in the
344 peripheral tissues which might minimize the possibility of interference by endogenous
345 vertebrate-like estrogen found in *Mytilus* (Reis-Henriques et al., 1990; Zhu et al.,
346 2003).

347 Our *in vitro* culture experiment showed that the presence of E_2 (10^{-6} M
348 concentration) in culture media is capable of upregulating the expression of MeER2
349 (ER) and its expression was also upregulated by T, which might be converted to
350 estrogens partially. Whereas no significant changes were seen in MeER1 (ERR)
351 indicating no response to neither E_2 or T, even though the concentrations were
352 relatively high compared to our previous *in vivo* experiments (Puinean et al., 2006;
353 Ciocan et al., 2010). In addition to the effect of E_2, the previous *in vivo* study reported
354 that a significant increase in MeER2 expression was observed the gonads in *M. edulis*
355 exposed to the E_2 (exposed to 5 - 200 ng/L) or synthetic estrogens (i.e. ethinyl
356 estrogen, EE_2 (exposed to 5 – 50 ng/L); estradiol benzoate, EB (exposed 200 ng/L)) at
357 the early stage of gametogenesis (Ciocan et al., 2010), assuming that E_2 might act for
358 MeER2 as a functional ligand. This E_2-dependent alteration for elevating ER
359 transcript level has already been confirmed in teleost species by showing that the
360 levels of three goldfish ER subtypes were differentially regulated by E_2 level (Marlatt
361 et al., 2008). Therefore, this autonomous regulation mechanism of ER transcription
362 may be present in *M. edulis* resulting in the increase of sensitivity to the possible
363 ligands. Further studies might be able to examine other vertebrate-type estrogens such
364 as estrone (E_1) and estriol (E_3) with E_2 at lower doses (e.g. 10^{-9} M and much lower) in

365 order to clarify whether this autonomous regulation is an E_2-specific response and
366 dose dependent. However this experiment could be difficult since their sensitivity can
367 be also affected by the endogenous estrogen-like steroid level that varies in
368 association with reproductive stages reported in scallop (Osada et al., 2004).
369 In addition to E_2-dependent disruption, the environmental exposure of *Mytilus*
370 to estrogens was not observed to change expression of vitellogenin (Puinean et al.,
371 2006), but could disrupt the expression of serotonin receptors (5-HT receptor) and
372 cyclooxygenase (COX) (Cubero-Leon et al., 2010). Nevertheless this variability of
373 MeER2 and other gene expressions in *Mytilus* induced by vertebrate-type estrogens,
374 their ligand specificity and affinity to MeER2 need to be further confirmed since this
375 study did not investigate this. By the sequential investigations from Thornton' group,
376 reporter or binding assays with vertebrate-type estrogens have accumulated evidence
377 that the mollusk ERs are constitutively active transcription receptors, meaning that the
378 mollusk ERs are ligand-independently regulated transcription factors (Bridgham et al.,
379 2014) but that annelid ERs are only activated by the vertebrate-type estrogen (E_2) in
380 the Superphylum Lophotrochozoa (Keay and Thornton, 2009). According to their
381 findings, *Mytilus* ERs might also behave as a ligand-independent transcription factors.
382 If so, the elevation of MeER2 expression with E_2 in this study would be explained by
383 the non-genomic response mechanism to estrogens (Canesi et al., 2004a; Canesi et al.,
384 2004b). They previously proposed the possible presence of an indirect pathway of E_2
385 signaling in *Mytilus* hemocytes which can moderate several kinase-mediated cascades
386 by changing phosphorylation state of transcription factors, also it was shown that ERs
387 are likely to exist in these hemocytes (Canesi et al., 2004a). Similar disruptive effects
388 were seen to occur in both *in vitro* and *in vivo* assays (Canesi et al., 2007b), as well as
389 in the digestive gland (Canesi et al., 2007a). These results in *Mytilus* could involve the
390 putative indirect auto-regulation mechanism of MeER2 through a non-genomic
391 pathway in gonadal cells as well.
392 In conclusion, this study first reports the molecular identification of full-length
393 coding sequence of an ER and an ERR in two *Mytilus* species. Our phylogenetic
394 analysis clearly visualize the genetic evolution of ERs and ERRs of *Mytilus* species.
395 The detection of ER and ERR transcripts in the *Mytilus* gonads with E_2-dependent
396 variability identified the different molecular behavior in response to vertebrate-type
397 steroids between ER and ERR, implicating the essential mechanism of ER in *Mytilus*

reproduction and that their gametogenesis are potentially disrupted by hormone-disrupting substances including vertebrate-type steroids.

Acknowledgements

We warmly thank Mr. Peter Darnell, Indian Point Mussel Farm, Nova Scotia, Canada, for courtesy of mussels. This work was supported by JSPS KAKENHI (23380109), Joint Project of Japan-UK Research Cooperative Program from JSPS and "Tohoku Ecosystem-Associated Marine Science (TEAMS)" research project funds from MEXT (to M.O.).

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536

537

Figure legends

Fig. 1 A comparison of protostome estrogen receptors.
(A) CLUSTALW alignment of amino acid sequences of estrogen receptors (ER/ER2) and estrogen related receptors (ERR/ER1). DBD; DNA-binding domain, LBD; ligand-binding domain. Amino acids conserved between species for a single type of receptor are colored in yellow or blue. Amino acids conserved between both types of receptor are colored in green. (B) Illustration of the MeER1 and MeER2 cDNA transcripts. The boxed region indicates regions that are translated to proteins. Letters A-E indicate the relative locations of the estrogen receptor domains. Species names and Genbank accession numbers: *Mytilus edulis*: MeER1 (**BAF34365**), MeER2 (**BAF34366**), *Mytilus galloprovincialis*: MgER1 (**BAJ07193**), MgER2 (**BAF34908**), *Capitella capitata*: *Capitella* ER (**ACD11039**), *Marisa cornuarietis*: *Marisa* ER (**ABI97117**), *Marisa* ERR (**ABI97120**), *Drosophila melanogaster*: *Drosophila* ERR (**NP648183**).

Fig. 2 Phylogenetic tree generated from the alignment of multiple estrogen receptors and estrogen related receptors. Sequences first described in this study are indicated by an asterisk (*). The human androgen receptor (Human AR) is included as an outgroup. Bootstrap values are indicated at nodes. Scale bare indicates an expected changes per site. Species names and Genbank accession numbers: *Thais clavigera*: *Thais* ER (**BAC66480**), *Nucella lapillus*: *Nucella* ER (**ABQ96884**), *Marisa cornuarietis*: *Marisa* ER (**ABI97117**), *Marisa* ERR (**ABI97120**), *Aplysia californica*: *Aplysia* ER (**NP_001191648**), *Octopus vulgaris*: *Octopus* ER (**ABG00286**), *Mytilus edulis*: MeER1 (**BAF34365**), MeER2 (**BAF34366**), *Mytilus galloprovincialis*: MgER1 (**BAJ07193**), MgER2 (**BAF34908**), *Crassostrea gigas*: Oyster ER (**BAF45381**), *Homo sapiens*: Human ER-alpha (**CAA27284**), Human ER-beta (**CAA67555**), Human ERR-alpha (**NP_004442**), Human ERR-beta (**NP_004443**), Human ERR-gamma (**NP996317**), Human AR (**ADK91081**), *Capitella capitata*: *Capitella* ER (**ACD11039**), *Drosophila melanogaster*: *Drosophila* ERR (**NP648183**).

Fig. 3 Representative tissue distribution of estrogen receptor transcripts in *Mytilus*. Black and gray bars show relative mRNA expression of MeER1 (A) and MeER2 (B), respectively in various tissues (gill, AM; adductor muscle, DG; digestive

gland, OV, ovary, TES; testis, SPENT; spent gonad, PG; pedal ganglion). Actin was used as an endogenous reference gene.

Fig. 4 *In situ* hybridization of estrogen receptor transcripts in *Mytilus* gonads and gills. Tissue type is indicated by rows and different probes are indicated by the columns. Sense strand hybridizations are shown as insets. OC; oocyte, FC; follicle cell, SC; Sertoli cell, CC; ciliated cell. Scale bars = 50 µm

Fig. 5 *In vitro* effects of estradiol-17ß and testosterone on estrogen receptor transcription in the *Mytilus* ovarian tissue cultured for two days. Dark and light gray bars show relative mRNA expression of MeER1 (A) and MeER2 (B) mRNA expression (n = 4 per treatment group, means ± S.E.). Tissue was treated with either estradiol-17ß (E_2) or testosterone (T) at 10^{-6} M concentration. An asterisk (*) indicates values that are significantly different from control ($p < 0.05$). E; estradiol-17ß, T; testosterone

Figure 1

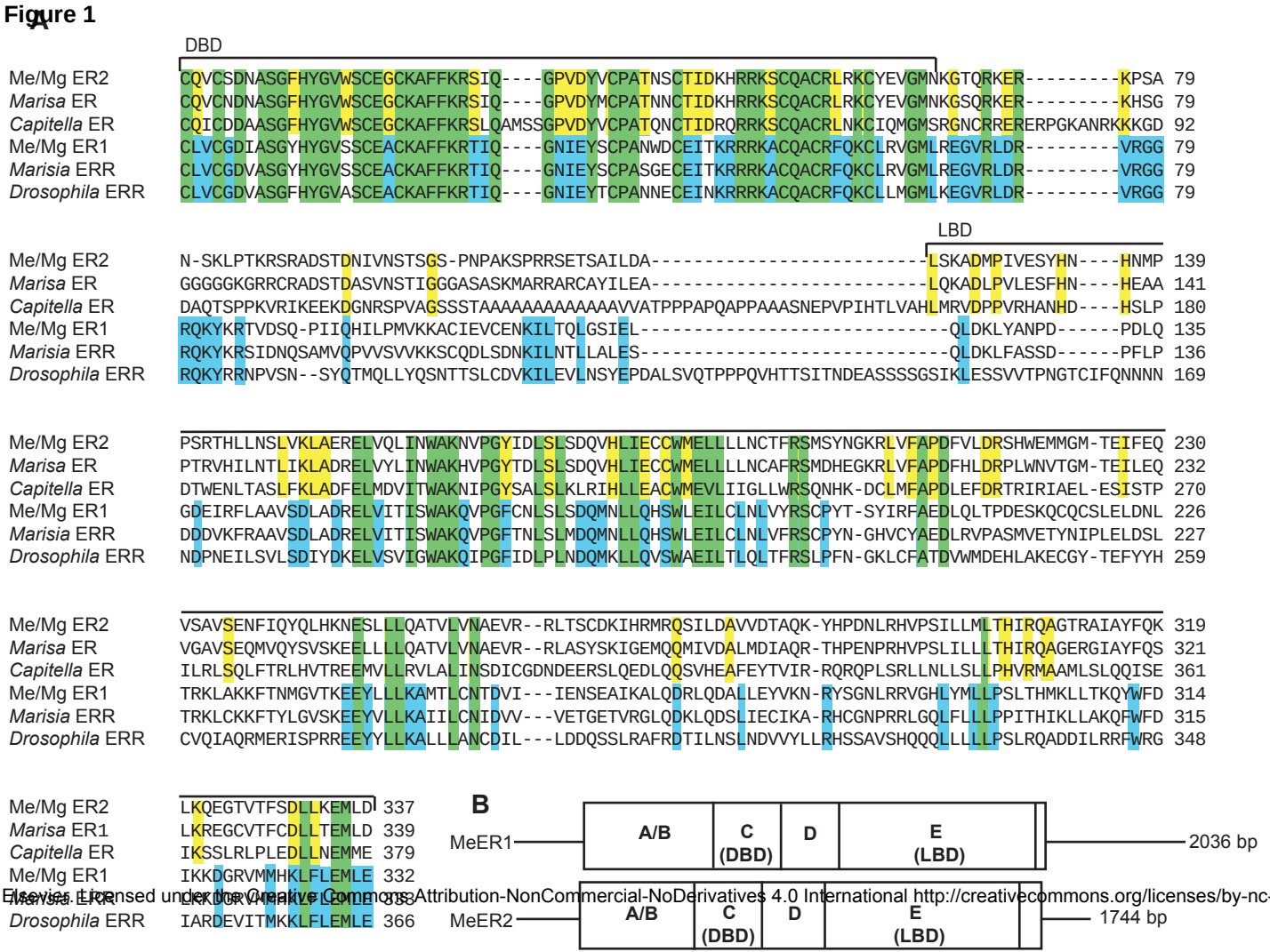

Figure 2

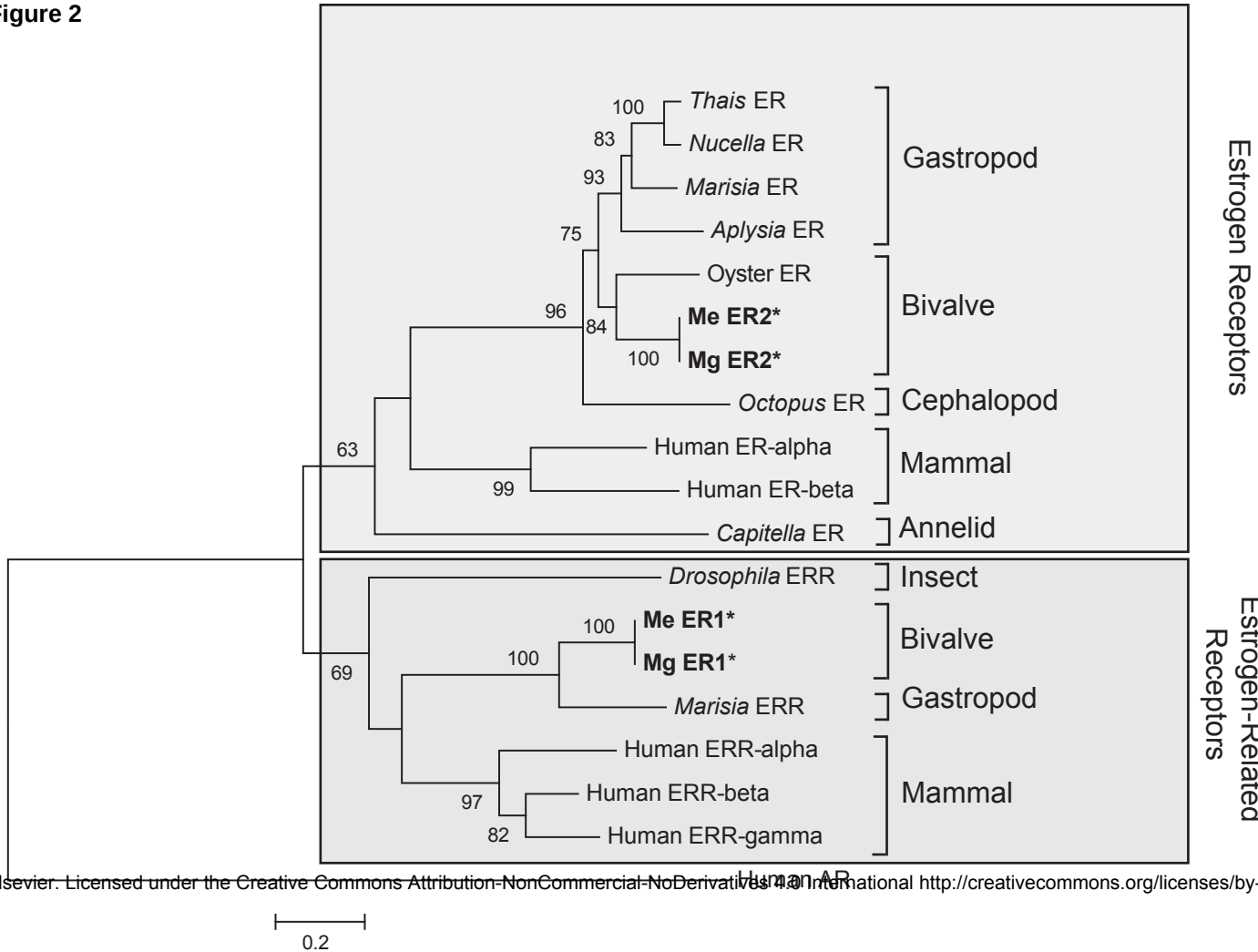

Figure 3

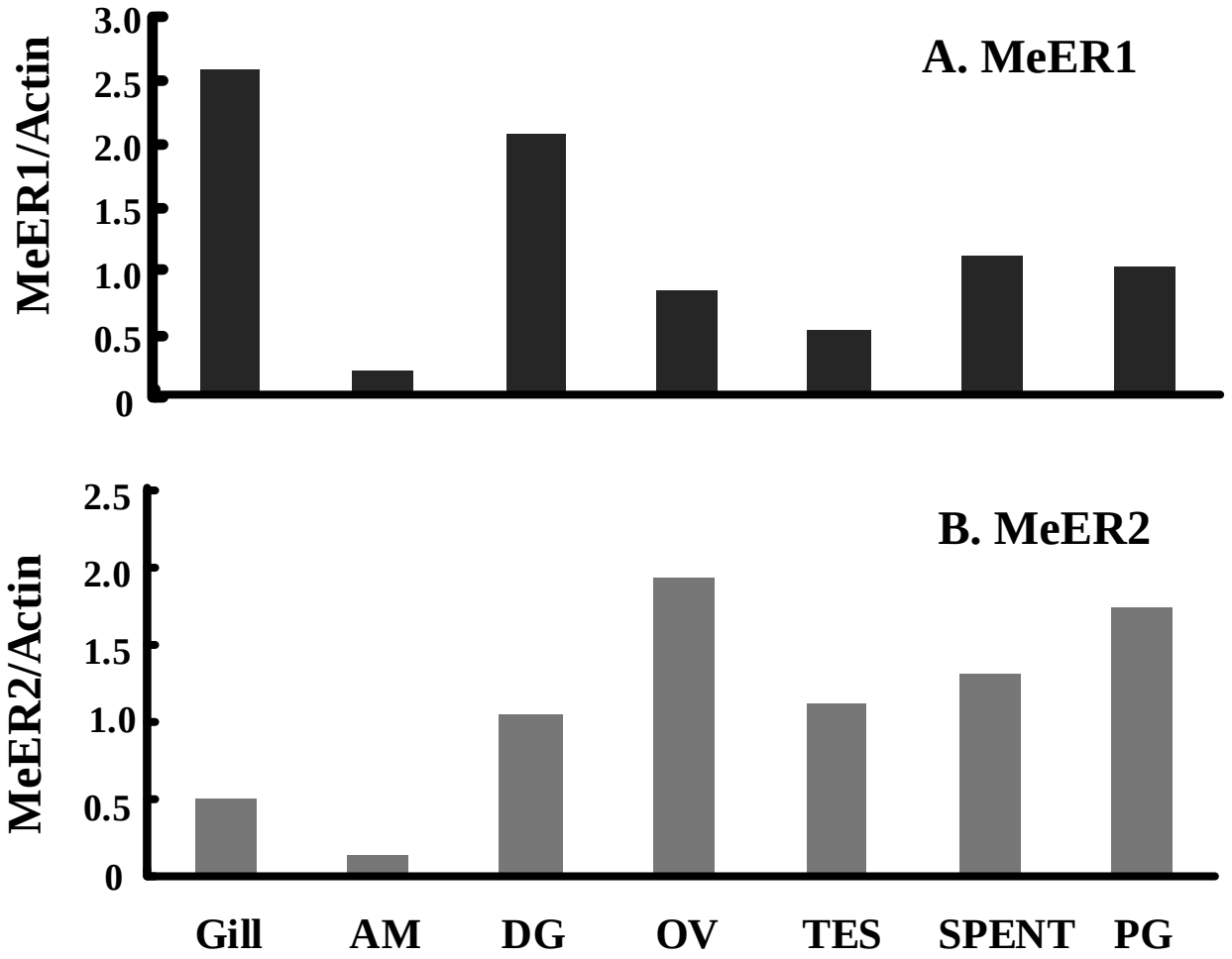

Figure 4

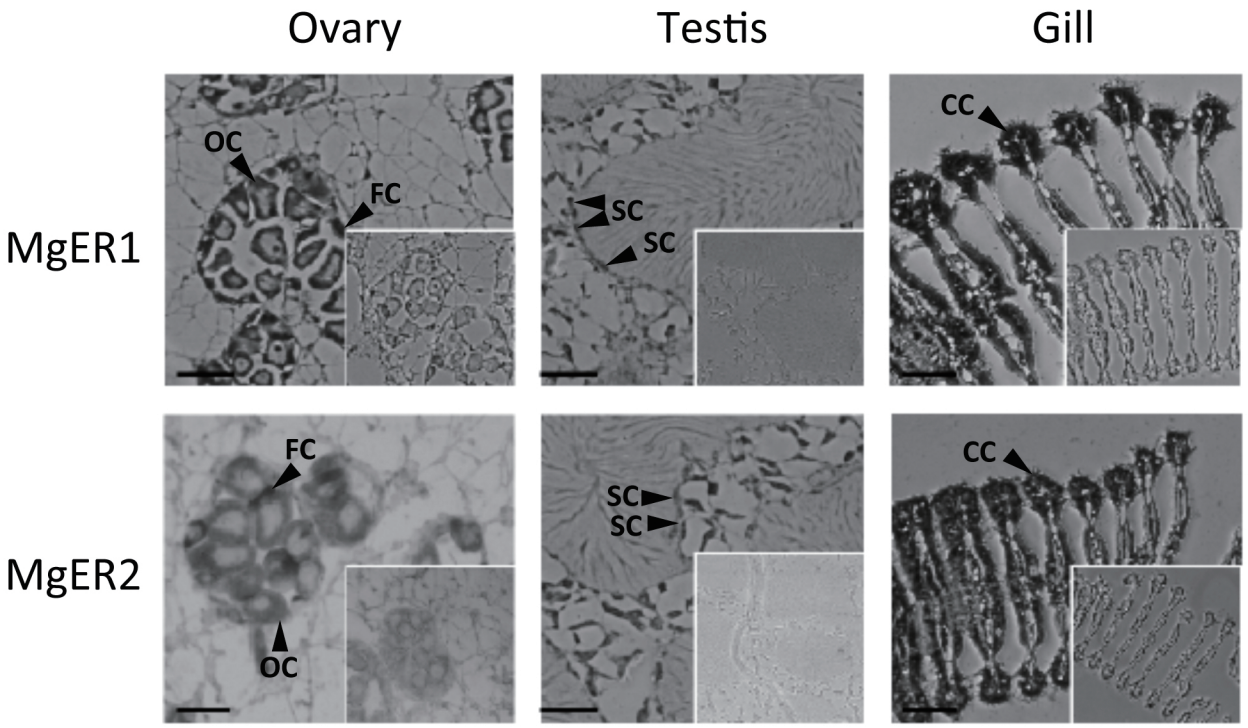

Figure 5