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THE DISCOVERY OF ESTROGEN RECEPTOR-BETA IN REPRODUCTIVE TISSUE OF MARINE POLYCHAETE, Perinereis nuntia BROODSTOCK

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Abstract: The polychaete *Perinereis nuntia,* a tropical segmented marine worm species in Thailand, has been cultured and wildly used in commercial shrimp live-feed, especially for the penaeid shrimp broodstock. Study of reproductive tissue of the marine worm is necessary to improve its culturing procedure. The presumption of this study is that the reproductive sandworm may generate and use estrogen to control inter-cellular group for reproductive functions as was reported in Korean lugworm, *P. aibuhitensis* and other vertebrate species. Actions of estrogen at its target cells are commonly known since it binds with the estrogen receptor (ER). Six brooders of female and male *P. nuntia* at 5-6 months of age and 3 pre-reproductive animals at 3-4 months old were examined for sites of ERβ protein synthesis and deposition by using indirect immunohistochemistry. A tissue distribution analysis of ER revealed positive immunoreactions in various tissues of the female and male broodstock, including cytoplasm of oocytes, cluster of cells at the parapodia and the intestinal mucosa corresponding to their reproductive age as was characterized by histological analysis. This report describes the first tissue-specific ERβ expression in *P. nuntia* broodstock. The expressed ER in reproductive tissues was likely related to the process of reproductive cell generation and the completion of reproductive cells of the polychaete.

Keywords: polychaete, *Perinereis nuntia*, reproduction, estrogen, estrogen receptor

INTRODUCTION

Due to the demand of fresh marine polychaetes for shrimp hatchery and baiting for sea fishing that has been progressively increased, the lacking of polychaete broodstock is subsequently occurred. Natural breeding of male and female brooders is depended on lunar cycle. In addition, polychaete in temperate zone would be not mating during winter. These items are a problem in limitation of juvenile polychaetes for commercial cultivation and farming. They are used as an aquaculture feed either live, in frozen meat, or as a constituent of formulated feeds as a maturation diet for shrimp broodstock (Olive, 1999).

A predominant species of polychaete, *Perinereis nuntia* found in Thailand has become importantly used for ovarian stimulation of the commercial penaeid shrimp broodstocks. Shrimp broodstock fed with live polychaetes are reported to have higher fecundity resulting in higher production of eggs and larvae (Withyachumnarnkul *et al*., 2002). Fresh polychaete meat contains high content of poly-unsaturated fatty acids, especially arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid (Luis and Passos, 1995; Graeve *et al*., 1997; Buhring and Christiansen, 2001) and other compounds including hormones.

Known hormone of polychaete that may play significant role in the reproductive process of crustaceans is prostaglandins (PGs) which derived from arachidonic acid (Spaziani, Hinsch

& Edwards, 1993). Prostaglandins F2a (PGF2a) play important role in the ovarian development and spawning in fish, crayfish and penaeid shrimp via the binding with cell surface G protein-coupled receptors (Fujino *et al*., 2000) and also through the nuclear receptors (Bhattacharya *et al*., 1999; Helliwell *et al*., 2004). In vertebrates, it is known that the estrogen plays significant roles in reproduction, development, growth, and sexual differentiation, exerting its actions through the ligand-induced transcriptional activation of the estrogen receptors (ERs) (Wallen, 2005; Heldring *et al*., 2007 and Nelson and Habibi, 2013). There are orthologues reports of ERs in many invertebrate species such as in mollusks (Kajiwara *et al*., 2006; Keay *et al*. 2006; Ni *et al*., 2013 and Nagasawa *et al*., 2015), amphioxus (Bridgham *et al*., 2008; Paris *et al*., 2008 and Katsu *et al*., 2010), and annelids (Keay and Thronton, 2009; Lv *et al*., 2017). Some trials have also traced for the up-regulation of invertebrate *ER* mRNA during exposure to the estrogen (Keay *et al*. 2006; Stange *et al*., 2011; Linlan *et al*., 2017). In addition, effects of estradiol-activated ERs on oogenesis and vitellogenesis have been continuously demonstrated (Li *et al*., 1998; Osada *et al*., 2003; Matozzo *et al*., 2008; Tran *et al*., 2016b). However, the functions of ERs and its mechanism of action in invertebrates are not well understood (Matsumoto *et al*., 2007; Bannister *et al*., 2007; Bridgham *et al*., 2008; Paris *et al*., 2008 and Katsu *et al*., 2010).

In vertebrates, differential expressions of ERβ and ERα in adult rat ovaries and testes by immunohistochemical assays have well demonstrated that both ER subtypes are important for reproductive functions and gamete production (Sar and Welsch, 1999; Hulas-Stasiak and Gawron, 2007). By biochemical, autoradiographic and immunohistochemical analyses, have also demonstrated the presence of ERs in brain, pituitary, mammary gland, prostate gland and reproductive tissues.

Recently, molecular characterization of full-length estrogen receptor gene and its tissue-specific expression in Korean lugworm, *P. aibuhitensis* was reported and revealed that the *paER* sequence is conserved among the annelid and mollusk species (Linlan *et al*., 2017). A tissue *paER* mRNA distribution also showed that it is strongly expressed in various tissues, including the intestines, stomach and esophagus.

In this study, we aimed to examine the tissue-specific for ER-beta (ERβ) protein expression in the polychaete, *P. nuntia* broodstock comparing to the juveniles by using immunohistochemical essay. The study of hormonal signaling for reproductive cells development and maturation is necessary to further improve a commercial polychaete culture.

MATERIALS AND METHODS

Polychaete

Experimental marine polychaete, *P. nuntia* broodstock (3 females and 3 males at 5-6 months of age) and 3 juveniles (approximately 3-4 months old) were obtained from the Coastal Aquaculture Research and Development Regional Center 2, Department of Fisheries, Samutsakhon province, Thailand. They originated from domesticated stocks and were reared in indoor cultured tanks containing artificial sea water at 10-15 ppt of salinity. They were maintained under prepared photoperiod (12h:12h of dark/light cycle) at 27-30 ^{o}C.

Histology

Polychaete cold-anesthetization, dissection, fixation with Davidson's fixative, paraffin embedding and tissue section procedures were based on method of Bell and Lightner (1998). Samples were various parts, including the head, body and tail regions. The specimens were sectioned at 5-6 μm thickness and placed on histological slides. The tissue sections were deparafinized with xylene, rehydrated, stained in Mayer's hematoxylin for 4-6 min, and passed through tap water for 5 min. They were then stained in eosin-phloxine 2 min, dehydrated, and

cleared in xylene. The H&E stained sections were mounted with coverslip and then examined under light microscope.

Immunohistochemistry

The parafinized tissue samples were sectioned at 5-6 μm thickness and placed on positively charged microscope slides (EMS, CA). Indirect immuno-labeling and immunohistochemistry were sent to carried out at the Pathology Diagnostic Center, Department of Pathology, Faculty of Medicine Siriraj hospital, Mahidol university with rabbit antiserum against ERβ subunit. For immunoperoxidase, biotinylated secondary antibody and HRP conjugate was applied before staining with DAB and H_2O_2 reaction, followed by hematoylin counterstaining. Mouse mammary tissue was parallely processed as a positive control section. The immuno-labeling sections were mounted with coverslip and then examined and photographed using light microscope.

RESULTS AND DISCUSSION

Presumption of this study is that the reproductive system of *P. nuntia* broodstock may generate and use estrogen to control inter-cellular groups for reproductive functions. Six brooders of polychaete (3 females and 3 males) at 5-6 months of age and 3 juvenile animals at 3-4 months old were examined by using histology and immunohistochemical assay for ERβ deposition.

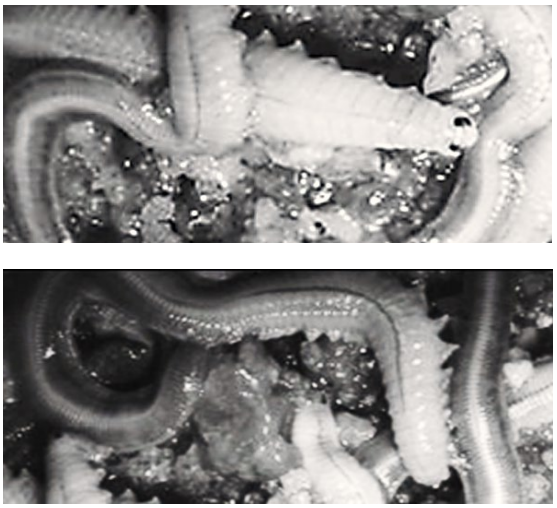

Figure 1. Photographs of *P. nuntia* broodstock, sexually mature (epitokous) polychaetes. Broodstocks are differentiated by the color and size of the proximal body region. Female (upper picture) being bright green colored with wider trunk, while males (lower picture) are creamy colored with narrower trunk

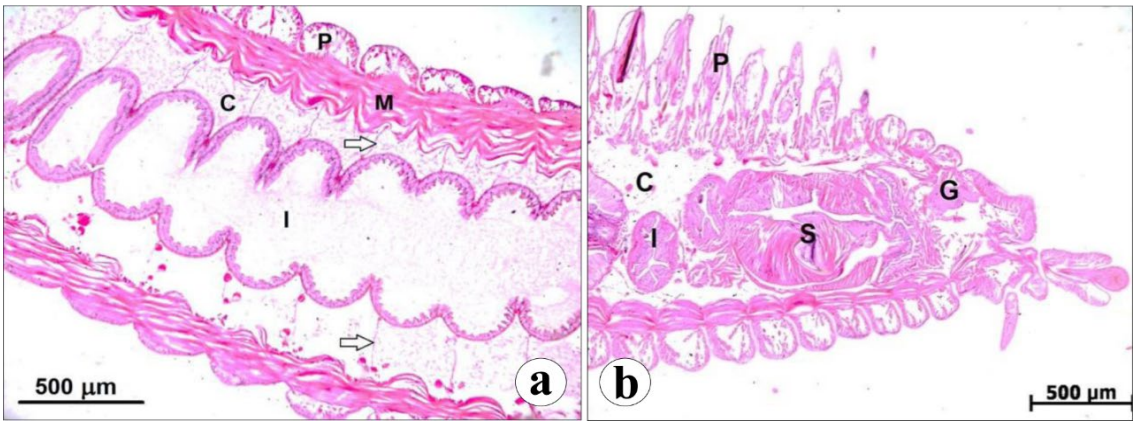

Figure 2. Photomicrographs of juvenile *P. nuntia* (3-4 months of age) stained with H&E of the body (a) and the head (b) reveal normal histology without deposition of gametes in the coelomic cavity (C). Intersegmental septa are observed between individual body segments (arrows). parapodia (P), muscular wall (M), intestine (I), stomach (S), cranial ganglion (G)

Histology with H&E stain showed accumulations of developing oocytes in the coelomic cavity within the head and the body of the female, and sperms in the male broodstocks (Fig 3). Results from histology were used to confirm the reproductive stage of the polycheates comparing with the pre-reproductive specimens that were studied.

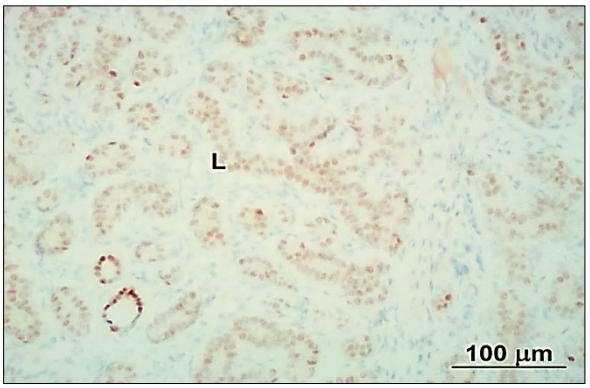

Figure 3. Photomicrograph of control immunohistochemistry using mouse mammary tissue exhibits positive reaction of anti-ERβ at the cytoplasm of epithelial cells of lactiferous gland as red-brownish color (L)

Indirect immunohistochemistry revealed positive reactions of anti- ERβ at the clusters of cells close to the tip of parapodia with all of female and male brooders as red-brownish color (Fig. 4). An intense immunoreaction also deposited at the cytoplasm of developing oocytes found within the coelomic cavity (Fig. 5). Mucosal cell layer of intestine of both the female and male broodstocks also showed positive reaction with ERβ subunit, whereas, non-immunoreactive signal was observed in juvenile specimens (Fig. 6). These features correspond to the reproductive phase of the polychaetes for eggs or sperms production and deposition found in the coelomic cavity. A control immunohistochemistry with mouse mammary tissue exhibits positive reaction of anti-estrogen receptor-beta (ERβ) at the cytoplasm of epithelial cells of lactiferous gland as red-brownish color (Fig. 7).

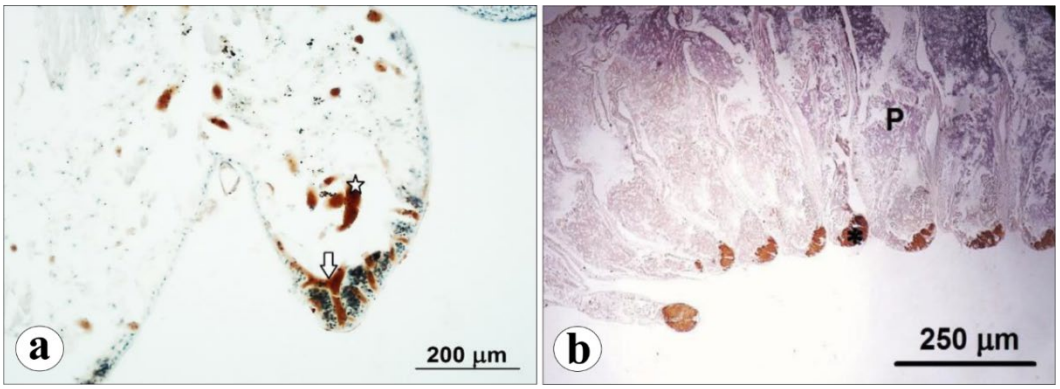

Figure 4. Photomicrographs of indirect immunohistochemical assay of female (a) and male (b) broodstocks exhibit positive reaction of anti-ERb at clusters of cells as red-brownish color (arrow, *) at the tip of parapodia. Oocytes or sperms are found within coelomic cavity as well as the cavity of parapodia (P). No positive immunoreaction was observed in cuticular wall and muscular tissue

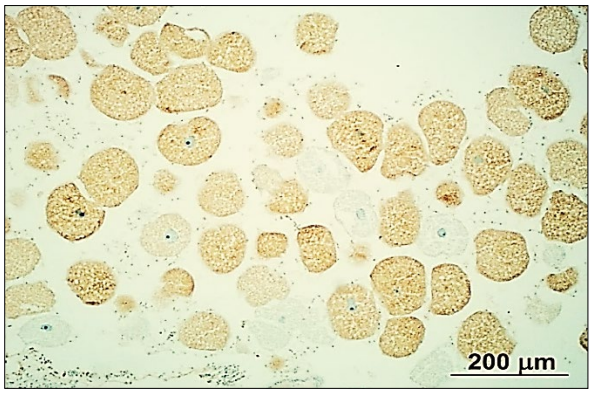

Figure 5. Photomicrographs showing ERb immunoperoxidase staining in the developing oocytes of female broodstock (5-6 months of age). ERb positive immunoreactions exhibit in the cytoplasm of the developing oocytes accumulated within the coelomic cavity

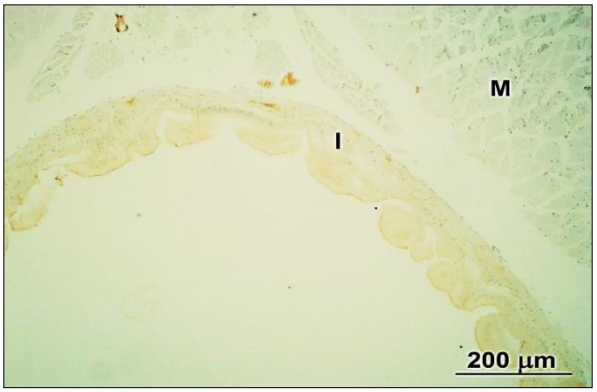

Figure 6. Photomicrographs showing ERb immunoperoxidase staining at the mucosal cell layer of intestine (I) of female broodstock. No positive immunoreaction was observed in the muscular tissue (M)

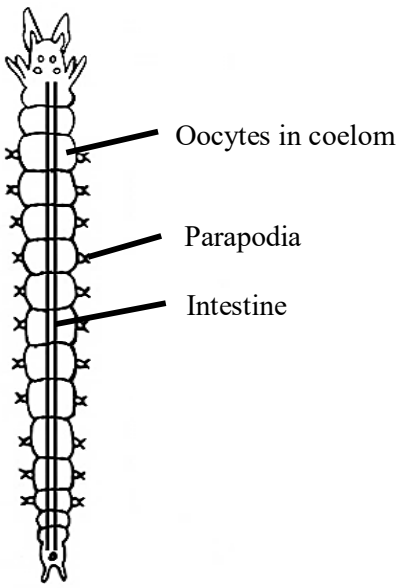

Figure 7. Drawing picture of *P. nuntia* broodstock demonstrates the cell and tissues mapping for ERb expression including oocytes in the coelomic cavity, the tip of parapodias and the intestinal wall

Monitoring of tissue specific location for synthesis and accumulation of estrogen receptor-beta (ERβ) protein in a marine polychaete, *P. nuntia* using immunohistochemistry described in this article revealed definitive tissues involved in the reproductive system such as cytoplasm of the developing eggs in the coelomic cavity of female broodstock, the intestinal mucosal cells and the cluster of germ cells at the tip of parapodia of both female and male broodstocks. Because *P. nuntia* is a fully segmented marine worms with body segments, its whole body cavities are containers with gamete production during the breeding season (Cho *et al*., 2015). Polychaetes are described as pseudohermaphroditic worm without permanent gonad (Rebscher *et al*., 2007). For instance in the localization of primary gonad of this worm, a molecular germline marker (*Pn-vasa*) sequence was applied to localize the primitive gonad and found that germ cells originate from the mesodermal posterior growth zone (MPGZ) at the tail region of the juveniles and then migrate into the anterior segments to form a transverse cluster of cells at distal end of parapodia of each body segments of the adult age (Maceren-Pates *et al*., 2015). This feature corresponded to the positive immuno-localization of ERβ in the *P. nuntia* broodstock. Unlike another well-studied annelid species, *Platynereis dumerilii* with incomplete intersegmental-septum, the gamete production mechanism was suggested that germ cells settled from the MPGZ at the tail region of the juvenile and later translocate into the coelomic cavity as a primitive gonad to develop oocytes (Rebscher *et al*., 2007). Result from tissue distribution analysis of *ER* mRNA in adult Korean lugworm, *P. aibuhitensis* showed the *paER* expressions in various tissues, including stomach, esophagus, esophageal gland, body wall, head, and most strongly in the intestines. (Lv *et al*., 2017).

In family Nereidae, the gonads have never become localized and oocytes grow freely in the female coelomic cavity. Vitellogenin, the precursor of yolk protein, is secreted by specialized coelomic cell called "eleocytes" and the intestinal mucosal cells during oogenesis in the King lugworm, *Nereis virens* (Garcia-Alonso and Rebscher, 2005). Vitellogenesis is the process of yolk formation in the oocyte or female germ cell and involved in reproduction of marine organisms. It starts when the fat bodies stimulates the release of juvenile hormones and

then produces vitellogenenin. A major role of the yolk proteins is involved in oocyte-sperm binding, sperm penetration and fertilization of zygote. An exogenous estradiol-17beta was used to demonstration on up regulation of the vitellogenin mRNA in *Xenopus laevis* (Skipper and Hamilton, 1997).

Polychaetes are used extensively for shrimp broodstock maturation diet due to their qualities in enhancing shrimp reproductive performances (Middleditch *et al*., 1980; Lytle *et al*., 1990). Such success partly results from their high-saturated fatty lipids (HUFAs) component, particularly arachidonic acid content as well as some reproductive hormones recently identified in polychaetes such as prostaglandin E2 (Meunpol *et al*., 2005b and prostaglandin F2α (Poltana, 2005). Other hormones discovered in polychaetes are ecdysteroid, osmoregulary hormones, oxytocin/vasopression hormones, reproductive hormones, sex hormones and sex pheromone. However, there is no evidence of vertebrate-type steroid identification in polychaetes (Meunpol *et al*., 2007).

Vertebrate-type steroids can be found in invertebrates such as androsterone, progesterone, estradiol, corticosteroids (Darvas *et al*., 1997; LaFont, 2000). Fingerman *et al*. (1993) reported that progesterone and estradiol are sex steroids important to the crustacean reproductive system and involve in ovarian development by stimulation of vitellogenesis and increasing oocyte diameter of *Penaeus vannamei*. A trial by using of 17α-hydroxyprogesterone extracts from natural *P. nuntia* and synthetic progesterone to induce development of the penaeid oocytes exhibited a significant increment of percentage of vitellogenic oocytes and reduce latent period of spawning (Meunpol *et al*., 2007).

The possibility of polychaetes possessing similar reproductive hormones to crustaceans especially vertebrate-type steroids is raised since polychaetes are acknowledged as being the best diet for shrimp maturation (Lytle *et al*., 1990, Browdy, 1992, Marsden *et al*., 1997 and Naessen *et al*., 1997). So, the identification of reproductive hormones as well as the receptors of these hormones in polychaetes can help in developing a reproductive maturation diet by combining these hormones into pellet food. In addition, a phylogenetic analysis of full-range *paER* gene exhibits orthologue of ERs among the annelids, crustaceans and other invertebrate species (Lv *et al*., 2017).

CONCLUSION

In summary, the present results describe the first tissue-specific ERβ expression in *P. nuntia* broodstock and indicate that the ERβ was exclusively present in the developing oocytes, intestinal mucosa and cluster of cells at the tip of parapodia of the reproductive worms, which equivalents to the location of primary gonads. On the contrary, ERβ localization was not observed in the juvenile specimens. These positive tissues of ERβ deposition probably mediate some of the effects of estrogen action in the regulation of growth and development of the gametes. Comparing to the results of immunohistochemical analysis of two ERs subtypes, ERβ and ERα in rat ovary, both ER subtypes are found in the oocytes, germinal epithelium and the granulosa cells of growing follicles, but no staining is detected in the primordial follicles as well as in the ovary of neonatal rat. However, the level of ERβ immunoreaction was higher in comparison with ERα. The exclusive presence of the ERβ in developing oocytes and granulosa cells in ovary indicates that the ERs mediates some effects of estrogen action in the regulation of growth and maturation of ovarian follicles and gametes during reproductive age (Sar and Welsch, 1999).

Polychaetes may need sex steroids at the specific time for reproductive purpose. To confirm this hypothesis, further studies need to examine the possibility of inducing oocyte/ sperm maturation and quantity through hormonal feed, as well as the time-course of ER expression during metamorphosis and growth of *P. nuntia*.

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