Marine Biotechnology
October 2011, Volume 13, Number 5, 971-980
http://dx.doi.org/10.1007/s10126-010-9361-4

A Functional Study of Transforming Growth Factor-Beta from the Gonad of Pacific Oyster *Crassostrea gigas*

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Abstract :

The transforming growth factor (TGF)-β superfamily is a group of important growth factors involved in multiple processes such as differentiation, cell proliferation, apoptosis and cellular growth. In the Pacific oyster *Crassostrea gigas*, the oyster gonadal (*og*) *TGF*-β gene was recently characterized through genome-wide expression profiling of oyster lines selected to be resistant or susceptible to summer mortality. *Og TGF*-β appeared specifically expressed in the gonad to reach a maximum when gonads are fully mature, which singularly contrasts with the pleiotropic roles commonly ascribed to most TGF-β family members. The function of og TGF-β protein in oysters is unknown, and defining its role remains challenging. In this study, we develop a rapid bacterial production system to obtain recombinant og TGF-β protein, and we demonstrate that og TGF-β is processed by furin to a mature form of the protein. This mature form can be detected in vivo in the gonad. Functional inhibition of mature og TGF-β in the gonad was conducted by inactivation of the protein using injection of antibodies. We show that inhibition of og TGF-β function tends to reduce gonadic area. We conclude that mature og TGF-β probably functions as an activator of germ cells development in oyster.

Keywords : Transforming growth factor-β – Bacterial expression – In vivo antibody inhibition – Reproduction – *Crassostrea gigas* – Marine bivalve

1. Introduction

The Pacific oyster *Crassostrea gigas* is prone to a complex summer mortality phenomenon, a major concern among oyster farmers (Samain et al., 2007; Samain & McCombie, 2008). Mortality results from interactions between environmental factors, pathogens and oyster physiological status (Samain and McCombie, 2008). Indeed, at the physiological level, reproduction in oysters is linked with summer mortality, and ripeness (the pre-spawning stage when oyster gonads are fully developed) appeared to be a critical period of vulnerability, mainly for oysters displaying high reproductive effort (Samain et al., 2007; Huvet et al., 2010). A recently conducted genome-wide expression profiling of oyster lines selected to be resistant (R) or susceptible (S) to summer mortality highlights reproduction and reproductive allocation as one of the principal pathways that play a role in resistance to summer mortality (Fleury et al., 2010). From the 34 ESTs reported to operate differentially between R and S lines, one EST corresponded to the gene oyster-gonadal Transforming Growth Factor-β (og TGF-β), a new TGF-β superfamily member (Fleury et al., 2008) Based on its tissue-specific mRNA expression in the germinal cells of both male and female gonads, og TGF-β was supposed to play a role in germinal cell proliferation and/or maturation (Fleury et al., 2008). In contrast, other members of TGF-β superfamily already identified in this model were ubiquitously expressed in all adult tissues and played a role in immunity or larval development (Herpin et al., 2004; Lelong et al., 2000, 2007). However, until now, no information had been obtained neither on the structure of og TGF-β protein nor on its function in oysters.

The TGF-β superfamily is a functionally diverse group of cytokines that regulate differentiation, proliferation, cellular growth, apoptosis or gene expression of several types of cell (Ten Dijke et al., 2000; Patterson and Padgett, 2000). A common feature of most TGF-β members is the presence of seven conserved cysteine residues, six of which form a typical cysteine knot structure, and one that forms the inter-subunit disulfide bond responsible for the covalent linkage of two subunits of the dimeric, biologically active ligand (Moore and Shimasaki, 2005). TGF-β superfamily members all possessed these conserved cysteine residues, but molecules are known by multiple names due to the circumstances of their discovery (Itman et al., 2006). Members of this superfamily have been further classified into several subfamilies: the TGF-β subfamily *sensu stricto*, an extensive bone morphogenetic protein (BMP) subfamily, the growth and differentiation factor (GDF) subfamily, the activin/inhibin subfamily, as well as several divergent factors (Knight and Glister, 2006). In vertebrates, TGF-β superfamily members are secreted by cells and interact with both type II and type I serine/threonine kinase receptors to transduce their signals through a signalling pathway involving specific Smad nuclear effectors that modulate gene expression and cellular properties (Ten Dijke et al., 2000; Massague and Wotton, 2000). Some members of the TGF-β superfamily exhibit specific roles in reproductive tissues by regulating the function of the ovary or testis (Josso and Di Clemente, 1999; Ingman and Robertson, 2002; Knight and Glister, 2006; Schulz et al., 2009). In mammals, TGF-β members act as paracrine factors, playing multiple roles in testis development (for a review, see Itman et al., 2006) or in ovarian functions such as stimulation of follicle development and maturation, steroid hormone production and gonadotropin sensitivity (Shimasaki et al., 2004; Knight and Glister, 2006). In lower vertebrates, some TGF-β members were reported to stimulate primordial germ cell and spermatogonial proliferation in rainbow trout (Sawatari et al., 2007) or to inhibit oocyte maturation in zebrafish (Kohli et al., 2003, 2005).

In the Pacific oyster *Crassostrea gigas*, *og TGF-β* mRNA is exclusively located in the somatic cells surrounding germinal cells in both male and female gonads (Fleury et al., 2008). Relative quantity of *og TGF-β* transcripts was seen to increase continuously during the development of the gonadal tubules and to achieve a maximal level when germ cells are fully mature (Fleury et al., 2008). In the present study, we develop a bacterial expression system

to produce the recombinant protein og TGF-β to gain information about its structure and function in the gonad of *C. gigas*. *In vivo* functional inactivation of mature og TGF-β using antibody injection was used to study the function of og TGF-β in the gonad of oysters.

2. Materials and methods

2.1. Bacterial expression of recombinant pro-og TGF-β

To produce pro-og TGF-β protein in bacteria, the pro-og TGF-β cDNA was inserted into an expression plasmid pGEX4 (GE Healthcare Life Science, Pittsburgh, PA, USA). This vector adds a GST-tag of 25 kDa at the N-terminus part of the recombinant protein that is fundamental for its successive detection and purification. The procedure for bacterial expression was done as described by Groisillier et al. (2010). Briefly, the plasmid carrying an inducible T7 promoter was transformed into *E. coli* host strain BL21 (Novagen, Gibbstown, NJ, USA) Then, in small-scale culture, the bacterial pellet was resuspended in 500 µl lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1 % Triton X100, DNase 1 mg/ml, lysosyme 1 mg/ml). Soluble and insoluble fractions were separated by centrifugation (12 000g, 20 min, 4°C). The insoluble pellet was resuspended in 200 µl lysis buffer supplemented with 6 M urea, and the soluble fraction was purified on Microspin GST purification columns (GE Healthcare Life Science, Pittsburgh, PA, USA) according to manufacturer's instructions. Samples from soluble and insoluble fractions were separated by 12 % sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In large-scale culture, the bacterial pellet was resuspended in PBS buffer (1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4; pH 7.3) containing 0.1 mg/ml of DNase and antiprotease cocktail (Complete EDTA-free tablets, Roche Applied Science, USA) and disrupted using a French press before centrifugation (12 000 g, 90 min, 4 °C). The resulting supernatant was applied onto a Glutathione Sepharose™ High Performance column (GE Healthcare Life Science, Pittsburgh, PA, USA) and the bound recombinant proteins were eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8). Results were analysed by 12 % SDS-PAGE electrophoresis. Protein eluted was then partially purified and concentrated using CENTRICON® centrifugal filters (Millipore, Billerica, MA, USA) according to manufacturer's instructions and protein concentration was estimated using a NanoDrop ND-1000 Spectrophotometer at 280 nm (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.2. In vitro thrombin and furin digestion

For thrombin digestion, 5 µg of eluted recombinant pro-og TGF-β was added to one unit of thrombin (Affinity Bioreagents, New England Biolabs, MA, USA) in PBS buffer. For furin digestion, 5 µg of eluted recombinant pro-og TGF-β was added to one unit of furin (Affinity Bioreagents, New England Biolabs, MA, USA), either in the manufacturer's recommended buffer (100 mM HEPES pH 7.5, 0.5% triton X100, 1 mM $CaCl_2$, 1 mM 2-mercaptoethanol) or a previously published furin buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM 2-mercaptoethanol; Leighton and Kadler, 2003). Furin digestion was processed at 20 °C or 37 °C from 5 min to 24 hours under rotative agitation. The digestion products were analysed by 12 % SDS-PAGE and detected by staining with Coomassie brilliant blue.

2.3. Tissue protein extraction

Gonads were isolated from a pool of 20 male or 20 female oysters of two-years aged grown in a hatchery under controlled conditions during five weeks and sampled at mature reproductive stage (stage 3) according to Fabioux et al. (2004). Tissues collected were immediately frozen and powdered in liquid nitrogen. Homogenized tissues (1 g) were solubilized in ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5 % Igepal, 2 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 100 mM

sodium fluoride, 10 nM sodium pyrophosphate, and 2 mM sodium orthovanadate ; pH 7.4) and proteins were extracted as described by Le Foll et al. (2007).

2.4. Western-blot procedures

Protein lysates were quantified using DC protein assay (Bio-rad, Hercules, CA, USA) and diluted at the same concentration in ice-cold lysis buffer. An equal amount of 20 µg of total protein extracts from gonad in each sample was denatured in Laemmli buffer before loading onto 15 % SDS-PAGE. One µg of the furin-digestion products of recombinant pro-og TGF-β was also denatured by Laemmli buffer and loaded in parallel with protein extracts from gonad. Proteins were then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Immunoblotting was performed using a commercially available heterologous pan-specific anti-TGF-β polyclonal antibody (AB-100-NA, RnD Systems, Minneapolis, USA; dilution 1:1000) as primary antibody and alkalin-phosphatase anti-rabbit IgG as secondary antibody (Bio-rad, Hercules, CA, USA; dilution 1/5000). Bands were revealed with a Immun-star AP detection kit (Bio-rad, Hercules, CA, USA) and band intensities were quantified by optical densitometry (Gel Doc XR scanning software, Biorad, Hercules, CA, USA). Dehybridization was done at room temperature for 1 hour by incubating the membrane in dehybridization buffer (100 mM glycine, 100 mM NaCl, pH 3.2) before immunodetection using anti-histone H3 antibody (#9715; Cell Signaling Technology, Danvers, MA, USA; dilution 1:5000) to verify that there was identical amount of total protein extracts from gonad loaded in each well, as described in Fabioux et al. (2009).

2.5. In silico analyses

Using InterProScan (Zdobnov and Apweiler, 2001), a consensus cleavage site, RFKR, was predicted in the C-terminal region of the protein of 307 amino acids encoded by the gene *og TGF-β*. Protein modeling of C-terminal mature og TGF-β was performed using SWISS-MODEL (http://swissmodel.expasy.org), then a diagram was produced using Swiss-Pdb Viewer (http://www.expasy.org/spdbv/; Arnold et al., 2006).

2.6. In vivo antibody injection and semi-quantitative histology

Twenty oysters of two-years aged were obtained from Marennes-Oléron (France) cultured stocks, and transferred to the Ifremer Laboratory in Argenton (France). They were acclimated for 1 week and maintained in raceways during eight days (T0 to T8) in conditions allowing optimal germ cell maturation (Fabioux et al., 2005). Oysters were injected at T0, T3 (3 days) and T7 (7 days) with a solution containing pan-specific anti-TGF-β polyclonal antibody (AB-100-NA, RnD Systems, Minneapolis, USA) diluted in PBS (n=10) or with the same volume of PBS (n=10). Direct injection in the gonad was demonstrated to be an efficient method in oyster (Fabioux et al., 2009). We therefore administrated the antibody by reitered injection directly in the gonad at 1.5 mg of antibody per kg for each injection, a dose used in vertebrate models by intraperitoneal injection (Ma et al., 2004). At T8, antibody-injected and control oysters were sampled, their gonads were immediately dissected and a transverse section of all the gonadic area was taken for histological examination of gonadic development according to Fabioux et al (2009). Only two different stages were observed in the present study, (3) ripe, (4) partially spent, according to the scale of Steele and Mulcahy (1999). Percentage areas of gonadic tubules, conjunctive tissue and digestive gland were then determined on each histological section. Slides were scanned with a digital scanner (HP scanjet 7400c) and the images saved in *.TIFF format. Tissue areas were then measured using image analysis software (Imaq Vision Builder, National Instruments Corp.). Gonad area percentage was estimated as pixel number, from gonad / pixel number on total sections, as described in Fabioux et al. (2005). Comparisons of the gonad area between conditions (antibody injection *versus* control) was performed by Student's t-test using STATGRAPHICS software after angular transformation of the percentages of gonad area.

3. Results

3.1. Production of recombinant pro-og TGFβ

To obtain the recombinant pro-og TGF-β protein, we cloned the *pro-og TGF-β* cDNA (Genbank accession number: EF563990; Fleury et al., 2008) encoding the pro-og- TGF-β lacking its signal peptide (Fig. 1 A) into the pGEX4-T1 expression vector. The GST-tagged pro-og TGF-β recombinant protein generated by this cloning strategy has a predicted molecular weight of 58 kDa (Fig. 1 B). Thrombin (Fig. 1 B, white arrow) liberates the 25 kDa fGST-tag and is expected to produce a 33 kDa fragment corresponding to pro-og TGF. If cleavage by furin (Fig. 1 B, black arrow) is effective, it is expected to produce two fragments, one of 13.6 kDa corresponding to mature og TGF-β and one of 44.4 kDa corresponding to GST-tagged pro-fragment .

After bacterial production, recombinant proteins were rapidly purified on Microspin GST purification columns and analysed by 12 % SDS-PAGE. By coloration, one major band was revealed at the molecular weight of approximately 58 kDa (Fig. 2 A, black arrowhead).This size is in close agreement with the molecular weight predicted from the amino-acid sequence of recombinant GST-tagged pro-og TGF-β inferred from the cDNA sequence in the expression vector. Since this band was also revealed by immunodetection using anti-GST antibodies (data not shown), our production protocol was validated and we carried-out large-scale production of this protein. As shown in Fig. 2 B, the synthesized 58-kDa protein was obtained in the soluble fraction of proteins although it was not detectable in insoluble proteins from bacteria pellet.. After HPLC purification, the synthesized GST-tagged pro-og TGF-β appeared as a 58 kDa band (Fig. 2 C) and was recognized by anti-GST antibody after immunodetection on western-blot (data not shown). The other upper band at 65 kDa (Fig. 2 C) was not recognized using GST antibody and was considered as a contaminant of the partially purified recombinant pro-og TGF-β. Several lower bands appeared at less than 30 kDa and were also considered as contaminants. Fractions eluted from HPLC containing GST-tagged pro-og TGF-β were then pooled, and recombinant protein was concentrated using centrifugal filters to obtain GST-tagged pro-og TGF-β at a concentration of approximately 0.1 mg/ml.

3.2. Cleavage of recombinant pro-og TGF-β by thrombin and furin

Although contamination of eluted GST-tagged pro-og TGF-β recombinant protein was evident, it was used for *in vitro* thrombin or furin digestion assays. A representative example of 12 % SDS-PAGE analysis of digestion products is shown in Fig. 3. The time course of digestion was examined by sampling 5 µl of digest products at 1 min, 30 min, 1 hour, 2 hours, 3 hours and 24 hours after enzyme addition at time 0. We show that the 58-kDa-band (Fig. 3 A, black arrowhead) disappeared rapidly after incubation with thrombin, confirming that this band contained a GST-tag. Indeed, thrombin digestion yielded two fragments, one corresponding to 33 kDa (Fig. 3 A, grey arrowhead), the size of pro-og TGF-β lacking its GST-tag, and the other to 25 kDa (Fig. 3 A, white arrowhead), the size of the GST-tag alone. These results demonstrate the integrity of recombinant GST-tagged pro-og TGF-β at a band of 58 kDa and illustrate the efficiency of its GST-digestion by thrombin. Using furin, the 58-kDa-band (Fig. 3 B, black arrowhead) corresponding to the recombinant GST-tagged pro-og TGF-β was partially digested. Two buffers and temperatures were tested and digestion was only partial but more effective over 24 hours after addition of the enzyme. The 58-kDa-protein was cleaved to yield a 44.4 kDa protein (Fig. 3 B, grey arrowhead) corresponding to the expected size of GST-tagged-pro-fragment liberated from the mature og TGF-β. The resulting mature og TGF-β migrating at the size of 13.6 kDa was not resolved on this 12 % SDS-PAGE.

3.3. Detection of mature og TGF-β in the oyster gonad

Using immunodetection on western-blot, we demonstrated that the commercially available heterologous pan-specific anti-TGF-β polyclonal antibody could recognize the recombinant mature og TGF-β of 13.6 kDa loaded onto a 15% SDS-PAGE gel. Using the same method, we analysed total protein extracts from gonad of male and female oysters sampled at the reproductive stage where gonads are fully mature (stage 3). Proteins were electrophoresed then transferred onto a membrane for immunodetection using pan-specific anti-TGF-β polyclonal antibody or a histone H3 antibody to control that equal amounts of gonad proteins had been loaded into the gel (data not shown). As shown in Fig. 4, the pan-specific anti-TGF-β antibody recognizes a band at 13.6 kDa corresponding to mature og TGF-β detected both in male and female gonad protein extracts (Fig. 4, M, F).

3.4. 3D structural prediction of mature og TGF-β

We analyzed the three dimensional structure prediction of the C-terminal mature og TGF-β of 13.6 kDa liberated by furin, to gain information about its structureby comparison with other biologically active TGF-β-related proteins. *In silico* analysis showed that the predicted structure of mature og TGF-β is built as a typical TGF-β superfamily member, and shares secondary-structure elements displaying a conserved α-helix and several β-strands that delineate two fingers (Fig. 5). By screening the available crystallographic structures of TGF-β members within the PDB protein data bank, we found that oyster mature og TGF-β three dimensional structure shares the highest identity score (23%; E-value of 1 10^{-35}) with the crystal structure of human bone morphogenetic protein BMP-2 (Fig. 5; structure ID: 3bmp; (Scheufler et al, 1999) that belongs to TGF-β superfamily. However, it does not contain the additional small secondary structure element (α-helix) in the loop region that is specific to the BMP sub-group (Fig. 5, asterisk), strenghthening its assignation as a member of TGF-β sub-group, as previously described by Fleury et al. (2008).

3.5. In vivo antibody injection in the gonad

To neutralize the function of mature og-TGF β, we injected the pan-specific anti-TGF-β antibody *in vivo* in the gonad of oysters maintained in food and temperature conditions known to stimulate oyster gonadic development. The injection was done three times during two weeks directly in the gonad to ensure a chronic neutralization. This antibody was chosen for its ability to neutralize the biological activity of several TGF-β in different species. At the end of the treatment, we performed histological analysis of gonadic tissue sections. From the 20 oysters conditioned, 19 were alive at the end of the experiment and 17 were analyzed for the gonadic area. Two oysters in the control condition were not considered in the present analysis due to figures of partial spawning observed on histological slides. Gonadic tissue surface percentages varied from 16.9 to 33.5 % for the oysters injected with the pan-specific anti-TGF-β polyclonal antibody, and from 26.8 to 51.1 % for the control oysters injected with PBS (Fig. 6). The data showed that oysters injected with antibody had a significant (P=0.003) lower mean gonad surface area (25.4 ± 5.8%) than control oysters (37.0 ± 7.9%) with an estimated mean difference of 11.6 %.

4. Discussion

Using the recombinant pro-og TGF-β, we demonstrated that furin (Leighton and Kadler, 2003)liberates a mature og TGF-β protein of 13.6 kDa as in vertebrates . Indeed, members of TGF-β superfamily are produced as inactive pro-protein and activation is linked to the elimination of the pro-domain by furin (Blanchette et al., 1997; Leighton and Kadler, 2003). Furin convertase liberates the biologically active TGF-β mature protein that can be recognized by specific transmembrane receptors to stimulate its intracellular signalling pathway (Cui et al., 1998; Chang et al., 2002). However, the cleavage of the pro-domain is necessary but not always associated with receptor signal transduction since mature TGF-β liberated from its pro-domain can also bind directly to the cell surface to stimulate its internalisation and degradation when it is not associated with its receptor (Lyon et al., 1997; Rider, 2006).

In oyster, we were particularly interested in examining three-dimensional structure of mature og TGF-β, since it contains only six cysteine residues including the fourth, but lacking the seventh characteristic residue (Fleury et al., 2008). By *in silico* analyses (Guex and Peitsch, 1997) we demonstrated that absence of the seventh cysteine does not interfere with the adoption of a typical TGF-β three dimensional conformation. Our data also demonstrate that mature og TGF-β is a protein that might be able to dimerize to exert its function in oystersince the common structure of oyster og TGF-β and human BMP-2 is the cysteine-knot motif and two finger-like double-stranded beta-sheets that are typical for TGF-β superfamily members known to dimerize (Scheufler et al, 1999; Herpin et al., 2004). Absence of the seventh cysteine was already reported for a member of TGF-β superfamily isolated from the gonad of rainbow trout, named Gonadal Soma-Derived growth Factor GSDF (Sawatari et al., 2007). These authors supposed that GSDF lacking this cysteine would not be able to form a dimer, and could thus function as a monomer, but these authors did not present any structure modeling or any evidence for monomeric or dimeric structure of the native GSDF.

Apart from a commonly pleiotropic role in several physiological processes, such as cellular proliferation, differentiation, matrix production and apoptosis (Massague et al., 2000; Ten Dijke et al., 2000), few components of the TGF-β system exhibit specific roles in reproductive tissues. However, a diverse range of activities for each of the TGF-β members in many aspects of reproduction has been suggested (Ingman and Robertson, 2002; Moore et al., 2004; Moore and Shimasaki, 2005; Knight and Glister, 2006; Mendez et al., 2006). In this study, we showed that the mature form of og TGF-β was recognized by the pan-specific anti-TGF-β antibody. This antibody was produced to be effective in a large range of species, from human to amphibian, and is directed against the C-terminal mature part of TGF-β (Ma et al., 2004; Hernandez-Pando et al., 2006). Because of the lack of proteomic tools to study protein of marine organisms, the choice of such an antibody was made on the basis of its recognition of mature TGF-β protein in multiple species. We compared the antigen of other commercially available anti-TGF-β antibodies with our amino-acid sequence and found that they did not match sufficiently to be effective in our model. The mature og TGF-β might thus be the *in vivo* functional factor as described for vertebrate homologues (Blanchette et al., 1997).

Using pan-specific anti-TGF-β antibody, we performed an *in vivo* antibody neutralization by injection directly in the gonad. Such *in vivo* immuno-inhibition studies are often used in vertebrate models (Sharma et al., 1996; Omer and Riley, 1998; Ma et al., 2004; Pinheiro et al., 2005) and the pan-specific anti-TGF-β antibody neutralizes the biological activity of TGF-β in many species (Biswas et al., 2006) to demonstrate its role in several physiological processes (Dumont and Arteaga, 2003; Subramanian et al., 2004; Pinheiro et al., 2005; Biswas et al., 2006; Hernandez-Pando et al., 2006). The same method was used to analyse the role of TGF-β in proliferation of ovarian somatic cells or differentiation of spermatocytes *in*

vitro (Gilchrist et al., 2004; Clelland et al., 2006; Mendez et al., 2006). In any case, such *in vivo* functional study offer an advantageous method for defining the role of a given protein in oyster. Improvement of this functional method must be done (dose and time-dependent effects, number and timing of injections, duration before sampling) with the aim of forcing the phenotype modifications between individuals, since a high variability of response was always observed in functional studies in oysters (Fabioux et al., 2009). Here, the entire og TGF- β was produced and the mature form was partially purified after *in vitro* digestion that was not very potent. Using the protocol detailed in this study, we can now emphasize the production of a recombinant mature og TGF- β that will be very helpful to obtain a specific antibody and better study the TGF- β expression in oyster.

Here we demonstrated that in the presence of neutralizing antibody, oysters presented a significantly reduced gonad area meaning a reduced number and/or size of gametes produced. Indeed, the percentage surface occupied by the gonad is directly linked to the number and/or size of gametes produced and is therefore representative of the reproductive effort of oysters (Royer et al., 2008; Huvet et al., 2010). A positive effect of og TGF-β is therefore suggested in germ cell development of oysters even though a negative effect was previously suggested for og TGF-β based on its over-expression in families displaying a reduced reproductive effort (Fleury et al., 2008). In any case, such *in vivo* functional study offer an advantageous method for defining the potent role of TGF-β in germ cell development in oyster. In vertebrates, some TGF-β members are potent stimulators of folliculogenesis and ovulation quota (Vitt et al., 2001; Knight and Glister, 2006) while other TGF-β members exert an inhibitory effect on gonad development (Moore et al., 2004). Some TGF-β superfamily members expressed in ovary and testis play a role as mitogens during folliculogenesis, stimulating proliferation of granulosa cells (Otsuka et al., 2001; Shimasaki et al., 2004). In contrast, in TGF-β 2-null-mutant mouse model, the number of germ cells increased in ovary (Memon et al., 2008). In lower vertebrates such as fish, some TGF-β members have an inhibitory effect on oocyte maturation in zebrafish (Kohli et al., 2003, 2005; Ge, 2005; Clelland et al., 2006) and steroid production in goldfish (Calp et al., 2003) while others can stimulate proliferation of primordial germ cells and spermatogonia in trout (Sawatari et al., 2007).

In conclusion, our work suggests that mature og TGF-β in the gonad acts as a stimulator of germ cell development in oyster. We clearly do not demonstrate whether mature og TGF-β is in monomeric or multimeric form to exert its function *in vivo* but we show that og TGF-β three dimensional structure might be compatible with dimerization. To gain insight into the molecular evolution of the structure-function of TGF-β superfamily, our future projects aim to obtain high-resolution detailed structure on the functional mature og TGF-β by determining its X-ray crystal structure. This work constitutes a major starting point to better understand an invertebrate TGF-β function in reproduction events in oysters.

Acknowlegments

The present research project was supported by "Europole Mer" (www.europolemer.eu; project "OxyGenes") and by ANR (project “Gametogenes”). The authors are grateful for the financial support from the ‘Marine Genomics Europe’ network of excellence (European Commission contract # GOCE-CT-2004-505403). E. Fleury was funded by Ifremer and a Région Basse-Normandie doctoral grant. We thank all the staff of the Argenton experimental hatchery for conditioning oysters. The authors are indebted to J.Y. Daniel, V. Quilien and C. Quéré for advice and technical assistance. We thank J. Moal for her comments on the manuscript and H. McCombie for editing the English language.

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Figures

Figure 1. Schematic representation of og TGF-β primary sequence.

(A) Schematic representation of the entire og TGF- primary sequence of 307 amino acids. Signal peptide (aa 1-18), pro-domain (aa 19-186) and carboxy-terminal mature og TGF-β (aa 187-307) are shown in black, grey and white, respectively. Partial sequence of the putative pro-domain cleavage site is shown below with the RFKR furin recognition site at Arg^{186} – underlined **(B)** Schematic representation of recombinant GST-tagged pro-og TGF-β corresponding to the protein deleted from its signal peptide and containing a GST-tag. Recognition sites for digestion by thrombin (white arrow) or furin (black arrow) are represented. Calculated molecular weight is indicated for each part of the protein.

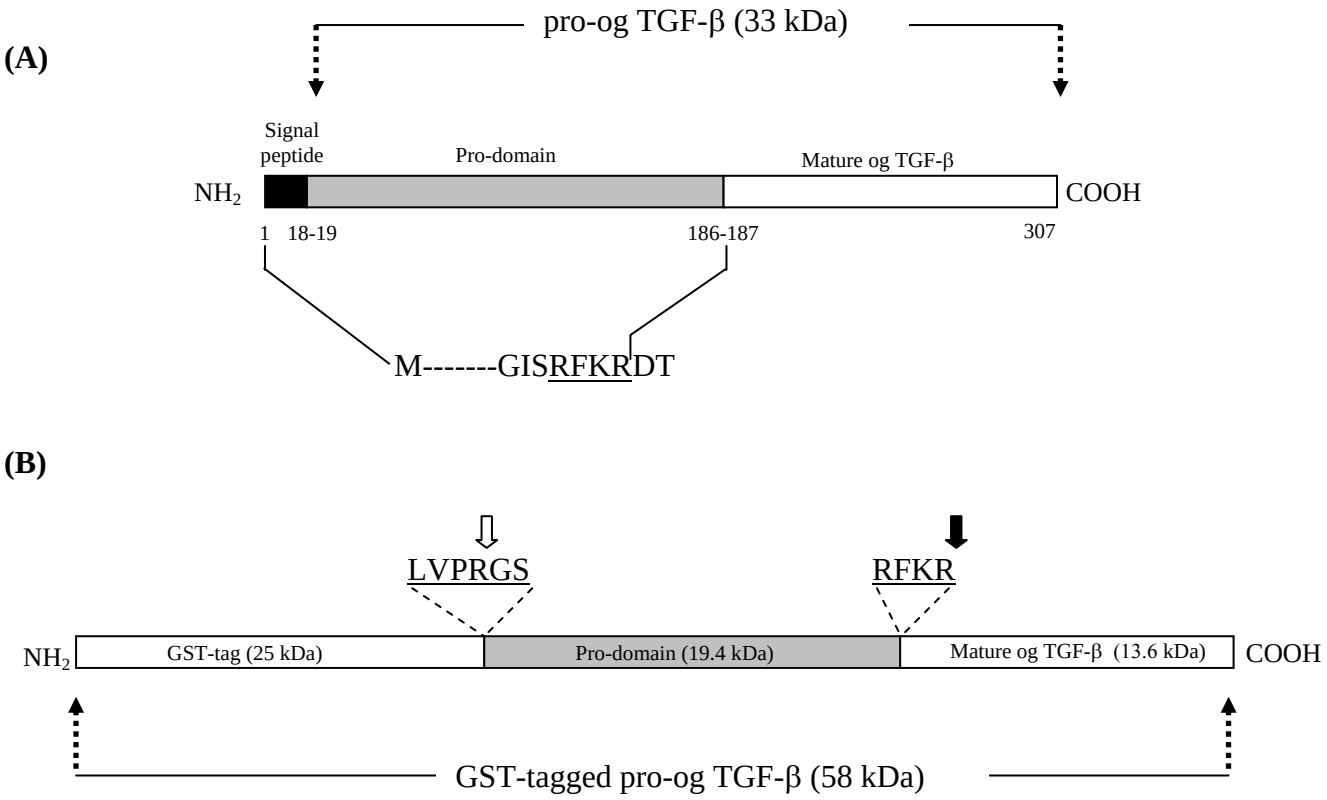

Figure 2. Expression of recombinant GST-tagged pro-og TGF-β

Protein separated on 10 % SDS-PAGE and detected by staining with Coomassie brilliant blue. **(A)** (M) Molecular weight markers. (1) Protein eluted after bacterial expression-test. (Black arrowhead) Band detected at 58 kDa corresponding to GST-tagged pro-og TGF-β. **(B)** Protein extracted from large-scale culture after French press disruption of bacteria. (I) Insoluble proteins contained in the bacteria pellet. (S) Soluble proteins. (Black arrowhead) Band detected at 58 kDa corresponding to GST-tagged pro-og TGF-β. **(C)** Representative SDS-PAGE of 3 fractions (1-3) collected by HPLC elution. (Black arrowhead) Band detected at 58 kDa corresponding to GST-tagged pro-og TGF-β. Sizes of marker proteins are given in the margin.

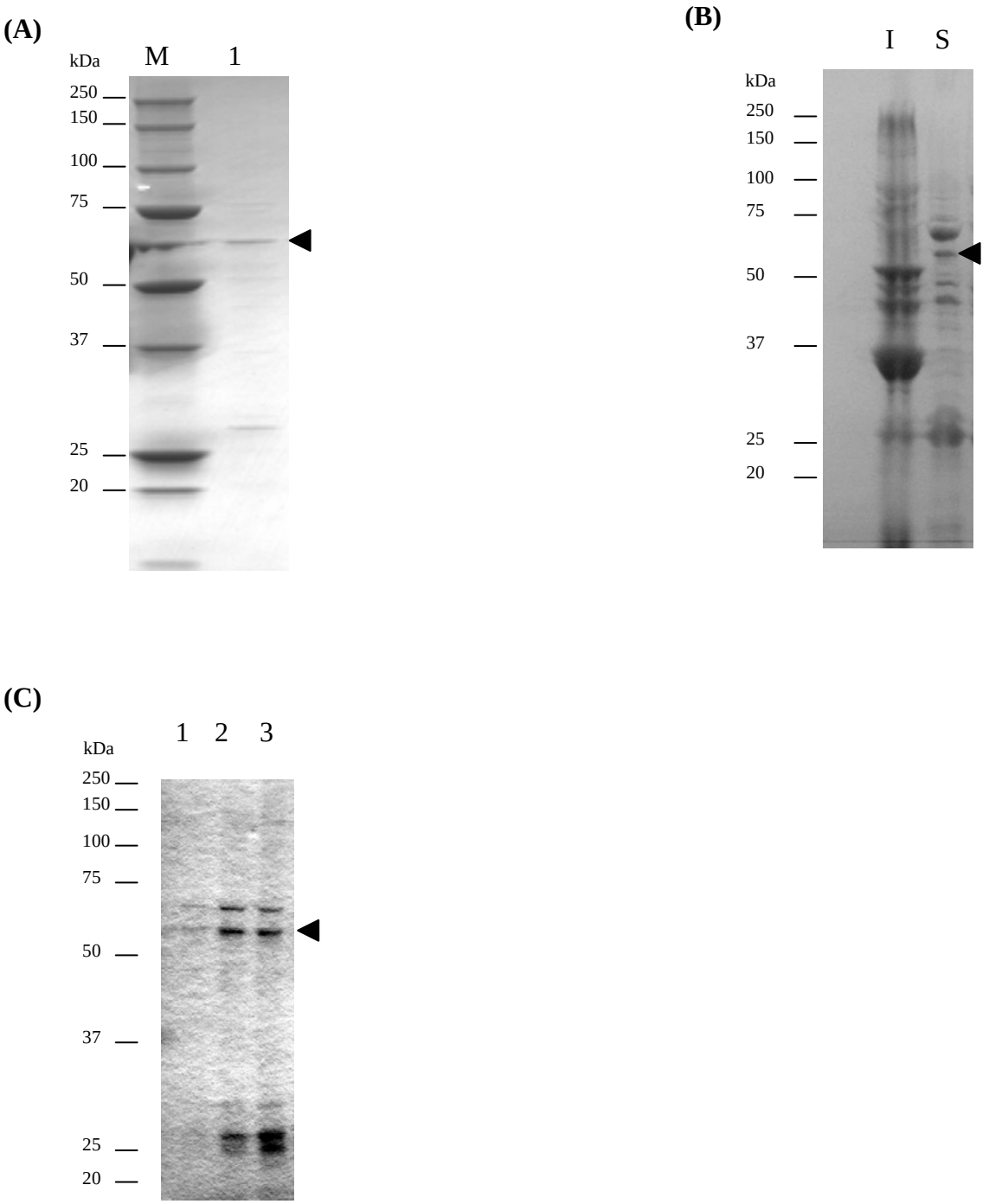

Figure 3. Digestion of recombinant GST-tagged pro-og TGF-β by furin or thrombin.

Digest products separated on 10 % SDS-PAGE and detected by staining with Coomassie brilliant blue. **(A)** Recombinant GST-tagged pro-og TGF-β was treated with thrombin (at 20°C) during 1 min (1'), 30 min (30'), 1 hour, 2 hours, 3 hours and 24 hours. (Black arrowhead) Band detected at 58 kDa corresponding to GST-tagged pro-og TGF-β. (Grey arrowhead) Band detected at 33 kDa corresponding to pro-og TGF-. liberated from GST-tag by thrombin. (White arrowhead) Liberated GST-tag detected at 25 kDa. **(B)** Recombinant GST-tagged pro-og TGF-β treated with furin (at 20°C or 37°C) during 1 min, 5 min, 1 hour, 2 hours, 3 hours and 24 hours. (Black arrowhead) Band detected at 58 kDa corresponding to GST-tagged pro-og TGF-β. (Grey arrowhead) Band detected at 42.4 kDa corresponding to GST-tagged pro-domain liberated from mature og TGF- by furin. Sizes of marker proteins are given in the left margin.

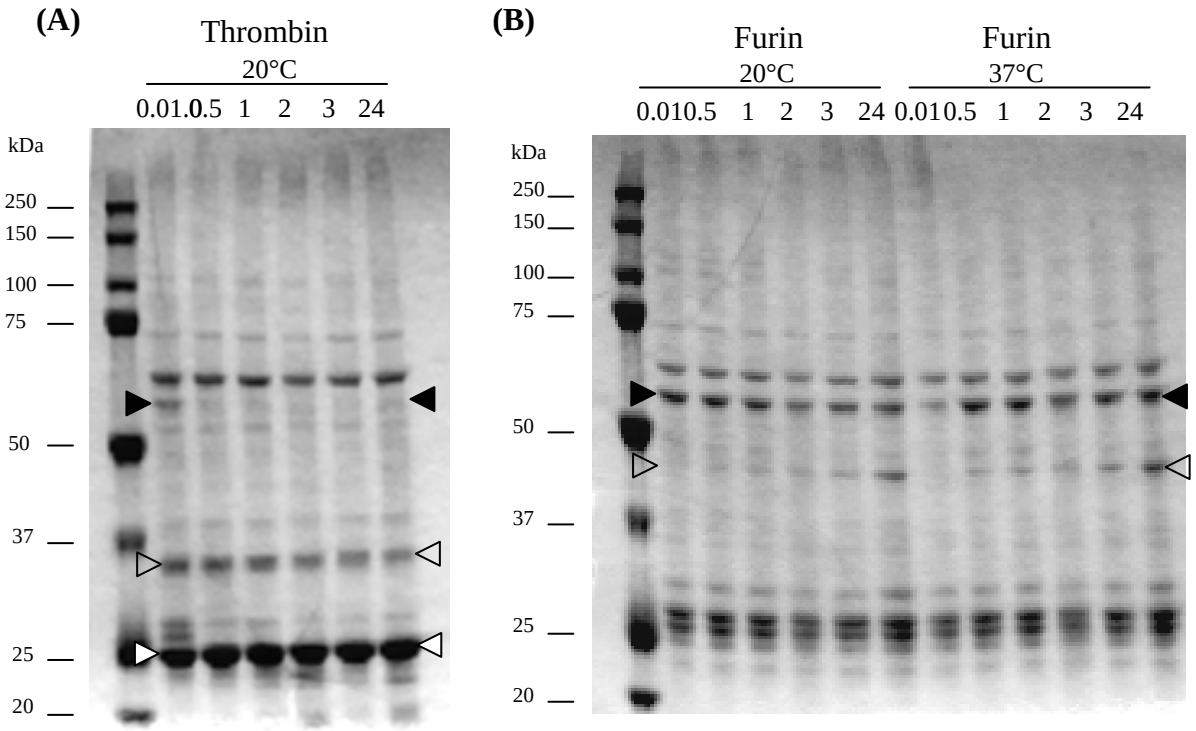

Figure 4. Immunodetection on western-blot using anti-TGF-β antibody.

Protein analysed by 15 % SDS-PAGE and immunodetection using heterologous pan-specific anti-TGF-β polyclonal antibody. The lower zone of the western-blot revealed by ECL is shown. (R) Recombinant mature og TGF-β obtained in furin digestion products. (M) Total protein extracted from male mature gonad. (F) Total protein extracted from female mature gonad. (Black arrowhead) Band of 13.6 kDa corresponding to mature og TGF-β recognized by the antibody. Sizes of marker proteins are given in the left margin.

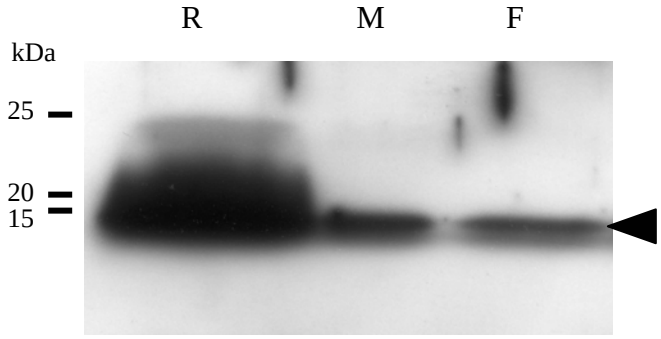

Figure 5. 3D structure modelling

Ribbon diagrams showing the tertiary structure of mature og TGF-β (oyster) compared to human BMP-2 (human): α-helices are indicated as spirals, β-strands as arrows. (Asterisk) Secondary α-helices structures specific to the BMP sub-group. Modelling was done using the SWISS-MODEL automated protein modelling server.

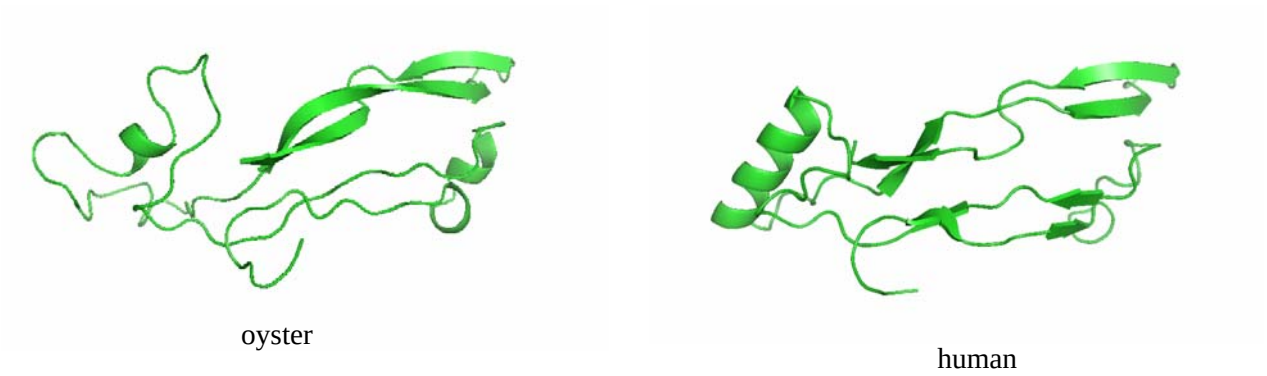

Figure 6. Quantitative histology

Mean reproductive effort estimated by the area occupied by the gonad (in %) for control oysters injected with PBS (black bar; control) and oysters injected with the pan-specific anti-TGF-β polyclonal antibody (gray bar; TGFb), n=10. Results are expressed as mean ± SE..