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Characterization of a gonad-specific transforming growth factor-β superfamily member differentially expressed during the reproductive cycle of the oyster *Crassostrea gigas*

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Abstract:

Through differential screening between oyster families selected for high and low summer survival, we have characterized a new transforming growth factor-β (TGF-β) superfamily member. This novel factor, named oyster-gonadal-TGFβ-like (og-TGFβ-like), is synthesized as a 307 amino acid precursor and displays 6 of the 7 characteristic cysteine residues of the C-terminal, mature peptide. Sequence comparison revealed that og-TGFβ-like has a low percentage of identity with other known TGF-β superfamily members, suggesting that og-TGFβ-like is a derived member of this large superfamily. Real-time PCR (RT-PCR) analysis in different oyster tissues showed that og-TGFβ-like is specifically expressed in both male and female gonads, at distinct levels according to the reproductive stage. Og-TGFβ-like relative expression was the lowest at the initiation of the reproductive cycle and increased as maturation proceeded to achieve a maximal level in fully mature female and male oysters. In situ hybridisation demonstrated that expression was exclusively detected in the somatic cells surrounding oocytes and spermatocytes. The role of this newly-characterized TGFβ member in the reproduction of cupped oyster is discussed in regard to the specificity and the localization of its expression, which singularly contrasts with the pleiotropic roles in a variety of physiological processes commonly ascribed to most TGF-β family members identified so far.

Keywords: Bivalve mollusc; Transforming growth factor; Gene expression; Oyster; Reproduction

Abbreviations: cDNA, complementary DNA; og-TGFβ-like, oyster-gonadal-Transforming-Growth-Factor-β-like; EST, expressed sequence tag; BMP, bone morphogenetic protein; SSH, suppression subtractive hybridization; DNase, deoxyribonuclease; RT-PCR, reverse transcriptase-polymerase chain reaction; ISH, in situ hybridization; UTR, untranslated region.

INTRODUCTION

Significant mortality has been reported in the Pacific cupped oyster, *Crassostrea gigas,* for many years (Cheney et al., 2000) and is a major concern of oyster farmers. Within the recently established, French national, multidisciplinary program “Morest”, set up to study the causes of summer mortality in *C. gigas*, divergent selection criteria based upon summer survival have been applied to produce resistant (R) and susceptible (S) oyster families (Dégremont et al., 2003, 2007). A previous analysis of the molecular events underpinning the physiological differences between R and S families was undertaken using suppression-subtractive hybridisation (SSH). This study supported the characterization of 46 differentially-expressed cDNAs between R and S families (Huvet et al., 2004).

With the aim of elucidating the molecular bases of summer survival, the present study reports the screening, using nylon array, of the 46 oyster SSH cDNAs from R- and S-selected families. Among the few differentially expressed genes characterized between R and S samples, one encodes a Transforming-Growth-Factor-β-related (TGFβ) protein. The TGFβ superfamily is a structurally conserved but functionally diverse group of widely distributed extracellular signalling proteins in metazoans (Herpin et al., 2004). On the basis of structural characteristics, members of this superfamily have been further classified into several subfamilies: the TGFβ *sensu stricto* subfamily, 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). The TGFβ superfamily members interact with both type II and type I serine/threonine kinase-specific receptors, which transduce their signals *via* the activation of Smad nuclear effectors (ten Dijke et al., 1996).

Members of the TGFβ superfamily are critical growth factors regulating a variety of important processes, such as proliferation and differentiation of several types of cells (ten Dijke et al., 2000) or acting during development. Apart from a commonly pleiotropic role in several physiological processes, a few key components of the TGFβ system exhibit specific roles, such as members implicated in testis (Itman et al., 2006) or ovarian follicle development (Knight and Glister, 2006) throughout the reproductive system of animals (Shimasaki et al., 2004). The various components of TGFβ signalling appear well conserved during evolution, but the repertoire of ligands is much larger in vertebrates than in invertebrates. In *C. gigas*, four ligands, one BMP/activin type II receptor, three subfamily type I receptors as well as most Smad downstream transducers, have been isolated (Herpin et al., 2002, 2004, 2005a, 2005b, Lelong et al., 2000, 2001,2007).

In the present paper, we report the characterization and the spatio-temporal expression of *oyster-gonadal-TGFβ-like* (*og-TGFβ-like*), a TGFβ superfamily member specifically expressed in the gonad of the cupped oyster, *C. gigas*.

1. MATERIAL AND METHODS

1.1 Biological material

For cDNA macroarray and real time PCR analyses, G2 and G3 oyster families were bred in March 2002 and 2003, respectively, at the Ifremer hatchery (La Tremblade, France) according to divergent-selection criteria (Dégremont et al., 2003, 2007). These oysters were then cultured at the Ifremer nursery (Bouin, France) where they were sampled. The remaining of G2 oysters stocks was transferred to Auray (South Brittany) and Baie des Veys (Normandy) sounds for a 12 months *in situ* rearing, where G2 R and S were sampled. The gonad was dissected from each

oyster, and pools of four gonads (3 pools R and 3 pools S per experiment) were prepared for total RNA extraction. For each experiment, the mortality peak was observed after the sampling (one or two weeks after). Four experiments are therefore available to generate expression profiles on R and S stocks: experiment (1) juvenile oysters, 4 month-old, G2, nursery; (2) juvenile oysters, 4 month-old, G3, nursery; (3) adult oysters, 16 month-old, G2, South Brittany field; (4) adult oysters, 16 month-old, G2, Normandy field.

For analysis of gene expression during embryonic and larval development, samples of various developmental stages, identified microscopically, were the same as those previously described in Fabioux et al. (2004a).

For analysis of gene expression in adult tissues, mantle, mantle-edge, gills, labial palps, gonad, striated muscle, smooth muscle, heart, haemocytes, and digestive gland were dissected from mature oysters and pools of 4 oysters (3 pools for each tissue) were prepared for total RNA extraction.

For analysis of gene expression during the reproductive cycle of *C. gigas*, samples were the same as those previously described in Fabioux et al. (2005). For each reproductive stage, gonads from 20 oysters were dissected for RNA extraction and histological analysis.

1.2 RNA and cDNA preparation

Total RNA was isolated using Trizol reagent (Gibco BRL) at a concentration of 1 ml/50 mg of tissue. Samples were then treated with DNAse I (1 U/µg total RNA, Sigma) to prevent DNA contamination. RNA concentrations were measured with a spectrophotometer at 260 nm using the conversion factor 1 OD = 40 µg/ml RNA, and RNA quality was checked by using the Bioanalyseur 2100 (Agilent). Reverse transcription (RT) was carried out as described in Huvet et al. (2003) using 2 µg total RNA from each sample.

1.3 Full length cDNA sequence

The full length sequence of the *oyster-gonadal-TGFβ-like* was obtained by overlapping three ESTs of *Crassostrea gigas*, one obtained from Marine Genomics Europe database (Tanguy et al., in press), a SSH-EST (**CK172353**, Huvet et al., 2004), and the last corresponding to the 5' extension of the SSH-EST. This extension was made by screening a cDNA library constructed in λ-ZAP II from *C. gigas* mantle-edge mRNA (Lelong et al., 2000), using a specific primer (5'-GAC ATC ATC TAC ACC CAG TTC CTG TC-3') and T3 universal primer. The amplified fragment was sub-cloned into pCR2.1® TOPO plasmid (Invitrogen), and sequenced (Qbiogene).

The full-length cDNA was amplified by PCR using primers designed at 5' and 3' extremities upon the contig of these 3 ESTs (forward: 5'-CAC GTG TAC CGT CAC CCT AT-3'; reverse: 5'-TGC AGT GGT TAG AAG CAA GG-3') with 0.5 unit of BD Advantage™ 2 polymerase mix (BD TITANIUM Taq DNA Polymerase, a proofreading polymerase and the BD TaqStart Antibody, Clontech). The amplified fragment was sub-cloned and sequenced.

1.4 Macroarray analysis

From the 46 differentially-expressed clones (Huvet et al., 2004), inserts were amplified by PCR using universal M13 primers and checked by electrophoresis. After denaturation (5 min at 95°C, one volume of 0.6N NaOH), each PCR product was blotted onto Hybond-N+ nylon membrane (Amersham) in duplicate using a Minifold I spot-Blot system, as well as oyster *actin* after PCR amplification. *Actin* was used as an internal control based upon its steady-state level of expression already tested between R and S families. Deionized water was blotted onto each nylon membrane as a negative control. DNA was cross-linked to the membrane at 70°C for 2 h. A

digoxigenin-labeled cDNA probe was synthesized from cDNA (10µg) for 2h at 37°C using the NonaPrimer kit (Qbiogene). The labelled probes were purified from unincorporated dNTPs using DNAprep resin. Membranes were prehybridized as described in Huvet et al. (2004). Hybridization was performed in duplicate overnight at 65°C in prehybridization buffer containing the denatured digoxigenin-labelled probe (20ng/µl). After hybridization, membranes were washed, as described by Huvet et al. (2004). The detection steps were performed according to the manufacturer's instructions (Dig nucleic acid detection kit, Roche Molecular Biomedicals).

The signal intensity was quantified using Multi-analyst software (Biorad), with the background signal removed. The value obtained is the spot intensity, expressed as an OD value per pixel, and multiplied by the spot-surface area. Values from 4 spots (duplicated membranes with duplicate spots) were used to calculate a mean value for each blotted cDNA. Transcript abundance was arbitrarily considered differential between R and S families when pooled values varied by greater than twofold.

1.5 Real-time PCR analysis

The level of four SSH-ESTs transcript was investigated by real-time PCR in the R and S samples analysed by macroarray using specific primers: **CK172362** (*oyster-gonadal-TGFβ-like*) forward: 5'-TTG GAC ATC AGG GAA ATT CTG-3', reverse: 5'-CCA AAC GAA ACG ACA GGA AC-3' ; **CK172358**: forward: 5'-CAA GAG CTT GGA CTT TGG GTA-3', reverse 5'-CAA AGA GCT ATG ACC GAG TGG-3' (Huvet et al., 2004) ; **CK172357** forward: 5'-CTG AAT GCA AAG AAA GGT TGG-3', reverse 5'-GAT CAT TGC ACA AAT CAC AGG-3' ; **CK172373** forward: 5'- ACA TCA GGT TTA CGG CGT TC-3' and reverse 5'-TGC CCA CCA ATA ACA ATG C-3'. The level of *oyster-gonadal-TGFβ-like* transcript was also investigated by

real-time PCR in oyster tissues and in gonad according to the reproductive cycle determined by histology using the same specific primers. Amplification of *elongation factor I* (*efI*) cDNA (Fabioux et al., 2004b) was performed to confirm the steady-state level of expression of a housekeeping gene, providing an internal control for gene expression.

The real-time PCR amplifications were carried out in triplicate as described in Huvet et al., (2004) with the iQ SYBR Green Supermix (Biorad) using an Icycler (Biorad). Each run included a positive cDNA control (one sample of the present experiment), negative controls (each total RNA sample with DNAse I treatment), and blank controls (water) analysed for each primer pair. PCR efficiency (E) was determined for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of cDNA to ensure that E ranged from 95 to 100 %. Relative expression of the target gene was calculated as $2^{(\mathrm{Ct}\ efI\ -\ \mathrm{Ct}\ og\text{-}TGF\beta\text{-}like)}$, with the cycle threshold of both genes at the same threshold limit.

1.6 In situ hybridisation (ISH)

Histology already has been published on the same samples (Fabioux et al., 2005). ISH was performed using sense and antisense DNA probes complementary to *og-TGFβ-like* cDNA according to Montagnani et al. (2001). *Og-TGFβ-like* insert was amplified by PCR using specific primers (forward: 5’- ACA CCC AGT TCC TGT CGT TT –3’; reverse: 5’- TGT GAT CCA GCA GAG AGA CA-3’).

1.7 Statistical analyses

Comparison of the relative level of *og-TGFβ-like* mRNA between R and S families was performed by Student's t-test using STATGRAPHICS software. Multiple comparisons of the

relative level of mRNA between tissues, reproductive stages and sexes were performed using one-way analysis of variance followed by multiple comparison test with the Tukey's HSD method using the same software.

2. RESULTS

2.1 cDNA macroarray analysis

The cDNA macroarray was hybridised by R and S samples collected in 4 experiments [(1) juvenile oysters, 4 month-old, G2, nursery; (2) juvenile oysters, 4 month-old, G3, nursery; (3) adult oysters, 16 month-old, G2, South Brittany field; (4) adult oysters, 16 month-old, G2, Normandy field] to focus on the genetic component rather than the effect of age, generation, or environment. This analysis revealed that 27 clones (of the 46 spotted clones) varied greater than twofold between the R and S progenies, in at least one of the 4 sampled experiments. Among these 27 clones, 4 ESTs appeared two-fold higher in R than in S families in the two conditions displaying significant mortality (experiments 1 and 2). Indeed, percentages of mortality for R and S progenies were, respectively, 4% and 74% for 4 month-old G2 oysters; 30% and 80% for 4 month-old G3 oysters. In adults, mortality appeared insignificant (mean= 8 ± 3%). By real time PCR, the relative mRNA level of these 4 ESTs appeared always significantly higher in R than in S progenies at the juvenile stage (experiments 1 and 2; Table 1). The comparison of those results with those obtained using macroarray shows that real time PCR analyses led to slightly under-estimation of the difference between R and S mRNA levels. However, both estimations appeared very well correlated (R^2= 0.82, p<0.001). Throughout the experiments, the mean ratio of *actin* mRNA level estimated by real time PCR was 1.02 and not significantly different between R and

S progenies (Table 1). From the sequences of these 4 ESTs, the blast search showed that 3 ESTs encode unknown proteins and one encodes a putative member of the TGFβ superfamily.

2.2 Isolation of the oyster-TGFβ-like cDNA from C. gigas

We obtained the full-length cDNA sequence of *oyster-gonadal-TGFβ-like* (Fig. 1, GenBank accession no. **EF563990**). The nucleotide sequence, which harbours untranslated 5' and 3' regions, encodes a preproprotein of 307 amino acids, starting with a predicted signal peptide of 18 residues. It contains a consensus cleavage site (RFKR) in position 183-186, which is conserved among all TGFβ superfamily members: *og-TGFβ-like* precursor is likely cleaved by a furin convertase at this typical proteolytic site to yield the C-terminal, mature peptide of 121 residues from position 187 to 307.

Alignment (Fig. 2) of mature Og-TGFβ-like with various TGFβ superfamily members shows that the sequence studied displays only 6 conserved cysteine residues and misses the seventh characteristic residue. The highest identity of Og-TGFβ-like mature region with other mature family ligands (Fig. 2) was observed with mammalian BMP 15 (32%).

2.3 Spatio temporal expression of the oyster-gonadal-TGFβ-like

The spatial expression pattern of *og-TGFβ-like* was analysed in 10 adult tissues (Fig. 3). A high relative level of transcript was detected in the gonad (mean relative expression ± SD = 0.028 ± 0.001); whereas, a weak or null signal was detected in the other tissues (0.0009 ± 0.0004). Relative transcript level detected in the gonad was approximately 30-fold higher than that observed in other tissues. The *elongation factor I*, used as a positive control, did not reveal significant differences between tissues at the 5% level.

In the gonad, the relative *og-TGFβ-like* transcript increased approximately nine-fold from stage 0 (mean relative expression of both sexes ± SD = 0.0033 ± 0.0002), where only a few germinal stem cells are present, to stage 3 (0.029 ± 0.00025) when gonadic tubules are filled with mature spermatozoa or oocytes (Fig. 4). Then, this mRNA decreased approximately by 3.5 until the next stage 0 (0.0088 ± 0.0012), in which oysters are partially spent with some residual germ cells, or totally spent. No significant difference of expression between the two sexes was observed at the 5% level.

RT-PCR analysis from unfertilised oocytes to trochophore stage of *C. gigas* did not reveal any detectable level of *og-TGFβ-like* transcript. This assay was repeated twice, in which *elongation factor I* mRNA level appeared at a standard level (data not shown).

2.4 Localization of oyster-gonadal-TGFβ-like mRNA by in situ hybridization (ISH)

As relative mRNA levels of *og-TGFβ-like* appeared the highest at stages 2 to 3 of gonad development (i.e. maturation step), localization of expression was assayed by ISH during the maturation of germ cells. Stage and sex were determined by histological observations (Fig.5 and 6, A, D).

At the beginning of the maturation step, the gonad of the female oyster contains tubule walls containing vitellogenic oocytes I, cells 30-45 µm in diameter with large nuclei and coarsely-granular cytoplasm (Fig. 5A). Conjunctive connective tissue is abundant. A strong ISH signal, corresponding to anti-sense probe hybridization, was detected in cells located between the vitellogenic oocytes (Fig. 5B and B'); whereas, no hybridization was detected in the germinal cells nor in the somatic cells of other tissues (haemocytes, conjunctive, or digestive gland cells). Later in the maturation stage, female tubules are filled with mature oocytes, and connective tissue

has almost disappeared. On the tubule walls, elongated cells, near oocytes appeared strongly-stained, and all tubule walls were also dark-blue stained, showing a strong *og-TGFβ-like* mRNA localization (Fig. 5D,E and E').

Concerning the male gonad, the beginning of the maturation step is characterized by tubules containing mainly stem cells, spermatogonia, and small, young spermatocytes (Fig. 6 A), respectively, from the outer wall to the centre of the tubule. ISH signal was observed at the periphery of the tubule. A higher magnification accurately localised *og-TGFβ-like* mRNA signal in the space between spermatogonia (Fig 6 B and B'); whereas, no staining signal was observed in germinal cells (stem cells, spermatogonia and young spermatocytes). When oysters were fully mature, lumina of male tubules were filled with spermatozoa, while germinal epithelia still showed all stages of male germ cells (Fig 6 D). The periphery of tubules corresponding to early stages of germ cells was stained with a pattern similar to that observed during the first steps of maturation. Spermatogonia were still unstained (Fig 6 E and E'). For all experiments, ISH controls using a sense probe did not generate any signal (Fig. 5 and 6, C and F).

3. DISCUSSION

3.1 The oyster-gonadal-TGFβ-like gene (og-TGFβ-like)

TGFβ members represent a widespread protein family in the animal kingdom, and some members have already been characterised in invertebrates. In *C. gigas*, two main members of this superfamily have been identified. The first, named mGDF, (Lelong et al., 2000) similar to human BMP2 and *Drosophila* DPP, is believed to play a role during metamorphosis. The second, Cg-TGF-β (Lelong et al., 2007), displays homology with both vertebrate TGF-βs and activins, and seems to regulate immune functions in molluscs. In this study, we report the characterisation of a

cDNA encoding a newly characterized TGF-β superfamily member, named *oyster-gonadal-TGFβ-like (og-TGFβ-like).*

A common feature of most TGFβ members is the presence of seven conserved cysteine residues, six of which form a characteristic cysteine knot structure and one forms the inter-subunit, disulfide bond responsible for the covalent linkage of two subunits of the dimeric, biologically active ligand (Moore and Shimasaki, 2005). The *Og-TGFβ-like* sequence has six conserved cysteines, including the fourth cysteine at position 271 which was shown for most members to be crucial for the formation of a functional, dimeric, mature protein (Daopin et al., 1992). As recently observed for teleost gonadal soma-derived growth factor (GSDF) implicated in proliferation of germ cells (Sawatari et al., 2007) this oyster sequence also lacks the seventh characteristic cysteine (replaced by a serine in position 306) involved in the cysteine knot structure.

Sequence and phylogenetic analyses (data not shown) revealed that *og-TGFβ-like* could not be certainty assigned to any of the known subfamilies. Although the closest homologue corresponds to BMP15, the percentage of identity between the mature region of the sequence studied with other related, mature ligands were always very low, suggesting that this newly-characterized *oyster-gonadal-TGFβ-like* gene is a derived member within the TGFβ superfamily.

3.2 Og-TGFβ-like expression during the maturation of the gonad

A high relative level of *og-TGFβ-like* mRNA has been detected exclusively in the gonad of both male and female oysters. This tissue-specific expression discriminates Og-TGFβ-like from other oyster TGFβ members, whose ubiquitous expression pattern suggests a pleiotropic role (Lelong et al., 2000, 2001, 2007). Moreover, the relative quantity of *og-TGFβ-like* transcript

increased continuously during the development of gonadic tubules in both sexes. Its maximum level was observed during active gametogenesis and, especially, when oysters were fully mature. A strong decrease in the relative *og-TGFβ-like* mRNA level was then observed when germ cells were expulsed; it appears significant when gonadic tubules contain only residual germ cells and regress. This expression pattern suggests a role of *og-TGFβ-like* in oyster gonadal development. In contrast to early embryonic expression of some TGF-β involved in primordial germ cell development, such as mammalian BMP4 and BMP8 (Chang et al., 2002), no significant *og-TGFβ-like* mRNA was found in any early oyster developmental stage (embryos and larva). This also contrasts with TGFβ members expressed in both developmental and reproduction stages, such as GSDF expressed during embryogenesis of the rainbow trout, and in the granulosa and Sertoli cells at later stages (Sawatari et al., 2007).

By ISH, *og-TGFβ-like* appeared exclusively located in cells surrounding oocytes in female and spermatogonia in males when germ cells mature; whereas, no staining was observed in the germ cells. The stained cells are elongated cells located around the tubule walls; they probably correspond to somatic cells, although these have never been described in *C. gigas*. In some invertebrates, ultrastructural studies describe somatic cells surrounding the germ cells, as "follicle cells" in the mussel (Eckelbarger and Young, 1999) or the sea spider (Miyazaki and Bilinski, 2006), or as "Sertoli cells" in the clams *Pitar rudis* and *Chamelea gallina* (Erkan and Sousa, 2002). They were found to form a close morphological association with developing germinal cells, as their cytoplasmic processes penetrate the germ cells, suggesting interaction between them (Erkan and Sousa, 2002). These cells are believed to contribute to the synthesis and transport of nutrients and regulatory substances to the developing germ cells, to phagocytosis of residual cytoplasmic bodies and degenerated germ cells, and also to serve as a mechanical

support for the development of germ cells (Hinsch, 1993). However, their function, in relation to regulation of spermatogenesis and oogenesis, remains to be demonstrated in molluscs. In *Drosophila*, the TGFβ signal-transduction pathway has been determined to affect germ-line stem cell number and the size of the stem cell niche (Schulz et al., 2004). This effect has also been observed for GSDF in the rainbow trout, which has a specific proliferation enhancing effect on the spermatogonia, and probably a maturation effect on the oocytes (Sawatari et al., 2007). The newly characterized member of TGF-β superfamily *Og-TGFβ-like* is, therefore, hypothesized to play a regulatory role in the differentiation and maturation of germ cells in the oyster *C. gigas* and constitutes a major starting point of further research on oyster reproduction.

3.3 Oyster-TGFβ-like is differentially expressed between summer mortality Resistant and Susceptible oyster families

Considering the hypothesized implication of *Og-TGFβ-like* in gonad development and germ cells maturation in *Crassostrea gigas*, the identification of such a factor in a genetic screening for summer survival between R and S families is intriguing. Summer mortality, however, occurs at the end of the maturation period and thus appears associated with reproduction (Worall and Widdows, 1984). In addition, significant differences in reproduction were previously observed between R and S families, in terms of gonad investment and spawning, suggesting that R families can survive summer mortality (especially in eutrophic environment) because they are not as reproductively active as S families (Samain et al., 2007). In that case, the two-fold higher expression of *og-TGFβ-like* in R families might suggest a negative effect in gonad development of oyster. This was already observed for some TGFβ members, such as TGFβ1, AMH or some BMP 15 in vertebrates (Kohli et al., 2005; Josso et al., 1998; Moore et al.,

2004) in opposition to other TGFβ members known as potent stimulator of the folliculogenesis and the ovulation quota in mammals (Vitt et al., 2001, Knight et al., 2006).

All these points underline the strong interest of developing functional analysis of the Og-TGFβ-like protein to establish its role in the reproduction of the oyster *C. gigas* and to test the hypothesis that reproductive effort may cause the higher rate of summer mortality of the selected, susceptible families.

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TABLES AND FIGURES

Table 1. Relative mRNA level of 4 ESTs between selected oyster families Resistant (R) and Susceptible (S) to summer mortality.

		Exp 1	Exp 2	Exp 3	Exp 4
Mortality rates		S: 74% R: 4%	S: 80% R: 30%	S: 14% R: 5%	S: 8% R: 5%
Accession number	Gene name	Fold change	Fold change	Fold change	Fold change
CK172362 / EF563990	*oyster-gonadal TGFβ-like*	**2.22** (1.97) *	**2.75** (2.52) *	1.24 (1.20)	**2.04** (1.43)
CK172358	unknown	**2.27** (1.99) *	**2.01** (1.51) *	1.52 (1.22)	**2.05** (1.59) *
CK172357	unknown	**2.26** (2.18) *	**2.16** (1.76) *	1.84 (1.59) *	**2.48** (2.35) *
CK172373	unknown	**2.28** (2.14) *	**2.10** (1.62) *	**2.18** (1.93) *	**2.02** (1.63) *
CK172378	*actin*	1.12 (1.02)	1.22 (1.00)	1.23 (1.03)	1.34 (1.05)

Expression profiles were generated by macroarray hybridized with R and S cDNA probes produced from 4 experiments and by real time PCR for the *oyster-gonadal-TGFβ-like* transcript. Underlined values: fold change varied greater than twofold in microarray ratio. Between brackets: real time PCR results, * significant at the 5% level using Student's t test. Exp: experiment (1) juvenile oysters, 4 month-old, second generation, nursery; (2) juvenile oysters, 4 month-old, third generation, nursery; (3) adult oysters, 16 month-old, second generation, South Brittany field; (4) adult oysters, 16 month-old, second generation, Normandy field.

Figure 1. Nucleotide sequence of the cDNA and deduced, amino-acid sequence of the *oyster-gonadal-TGFβ-like* gene from the Pacific oyster *Crassostrea gigas*.

The predicted segment for the signal peptide is underlined. The arrow indicates the putative site of cleavage of the signal peptide. Grey highlighted sequences are the two potential sites of N-glycosylation [NX(S/T)]. The box shows the consensus sequence of proteolytic cleavage. The 6 conserved cysteines characteristics in TGFβ are bold. Accession number: **EF563990**.

Figure 2. Alignment of the deduced, amino-acid sequence and percentage identity of the *oyster-gonadal-TGFβ-like* with other sequences of TGFβ of different species.

Alignment was generated from the first conserved cysteine residue by using CLUSTAL W. Amino acids identical between *oyster-gonadal-TGFβ-like* and other members are indicated in black and amino acids with similar physiochemical nature in grey. Asterisk indicates the 7 conserved cysteines for most of the TGFβ members (with the missing seventh cysteine in *og-TGFβ-like* in grey). Points denote gaps introduced to maximize the alignment. Percentage of identity was calculated using DNAMAN.

Cg: *Crassostrea gigas*, Ss: *Sus scrofa*, Hs: *Homo sapiens*, Dm: *Drosophilia melanogaster*, Dr: *Danio rerio,* Xt: *Xenopus tropicalis,* Hv: *Hydra vulgaris,* Pm: *Petromyzon marinus,* Pf: *Pinctada fucata,* Oc *: Oncorhynchus mykiss*

Figure 3. Level of the *oyster-gonadal-TGFβ-like* transcript relative to *Elongation Factor I* transcript in tissues of wild mature *Crassostrea gigas* oysters analysed by real-time PCR. Bars represent standard deviation at 5% level. Significantly different means ($P<0.05$) are indicated by letters.

Figure 4. Level of the *oyster-gonadal-TGFβ-like* transcript relative to *Elongation factor I* transcript analysed by real time PCR in male and female gonad during a complete annual

reproductive cycle of *Crassostrea gigas*. Light grey bar: undifferentiated, dark grey bar: female, white bar: male. Stage 0: resting period, stage 1: initiation, stage 2: maturation, stage 3: ripeness , stage 4: post-spawning and stage 0': regression and next resting period. Bars represent standard deviation at 5% level. Significantly different means (P<0.05) are indicated by letters.

Figure 5. Histological analysis and *oyster-gonadal-TGFβ-like* expression by ISH in female *Crassostrea gigas* gonad.

A, D : Histological analysis. A : maturation step, D: end of the maturation step. B, C, E, F: The *oyster-gonadal-TGFβ-like* mRNA in female *C. gigas* gonad stained by *in situ* hybridization with an antisense probe. Positive cells are stained in dark blue. B: maturation step, C: negative control of the maturation step (sense probe), E: end of the maturation step, F: negative control of the maturation step (sense probe). Ct: conjunctive tissue, Ooc: oocytes, Mooc: Mature oocytes. Scale bar: 15 µm; magnification: 200x for each panel, except for A', B', D' and E': 2000x.

Figure 6. Histological analysis and *oyster-gonadal-TGFβ-like* expression by ISH in adult male *Crassostrea gigas* gonad.

A, D: Histological analysis. A : maturation step, D: end of the maturation step. B, C, E, F: The *oyster-gonadal-TGFβ-like* mRNA in male *C. gigas* gonad stained by ISH with an antisense probe. Positive cells are stained in dark blue. B: maturation step, C: negative control of the maturation step (sense probe), E: end of the maturation step, F: negative control of the maturation step (sense probe). Ct: conjunctive tissu, Spg: spermatogonia, Spc: spermatocyte, Spz: spermatozoa. Scale bar: 15 µm; magnification: 200x for each panel, except for A', B', D' and E': 2000x.

Figure 1

```
cacgtgtaccgtcaccctatttaacatcagtgttggtaacctagtgttgaccctctgtttatataaaatgaaacattttcaaat    84

                                                  M   R   S   S   G   I   F   L          8
cacattccaaacatcacgaatactttatcatagaatcatgggcttgtta ATG AGA TCA AGT GGC ATT TTT TTA      157

 M   F   C   V   Y   C   F   V   N   G   G   P   L   F   S   D   E   M   L   N   M      29
ATG TTT TGT GTA TAT TGT TTT GTG AAT GGT GGA CCA TTG TTC TCA GAT GAA ATG CTT AAC ATG    220

 G   N   S   F   T   K   D   L   P   L   S   M   Q   R   L   L   D   K   F   M   E      50
GGT AAC TCC TTC ACA AAG GAC CTT CCT TTG TCT ATG CAA CGT TTA CTG GAC AAA TTT ATG GAG    283

 S   V   D   A   I   N   S   D   E   N   S   D   I   S   F   H   S   L   E   N   V      71
TCC GTA GAT GCC ATT AAC AGT GAT GAA AAT TCC GAT ATT TCC TTT CAT TCA TTA GAA AAC GTG    346

 N   G   G   Y   E   L   D   I   R   E   I   L   A   D   H   D   V   T   G   V   D      92
AAT GGT GGG TAT GAA TTG GAC ATC AGA GAA ATT CTG GCG GAC CAT GAC GTC ACT GGG GTG GAT    409

 L   I   M   T   S   S   T   P   S   S   C   R   F   V   W   V   E   N   D   I   K     113
CTC ATA ATG ACA TCA TCT ACA CCC AGT TCC TGT CGT TTC GTT TGG GTG GAA AAT GAC ATC AAA    472

 L   D   G   R   R   I   N   E   Y   V   F   F   N   I   D   H   L   L   A   M   E     134
TTG GAT GGT AGA CGC ATC AAC GAA TAC GTT TTC TTT AAT ATT GAT CAT CTA CTG GCA ATG GAA    535

 H   S   S   M   L   S   T   I   R   I   V   P   S   S   D   C   F   R   Q   H   E     155
CAT TCC TCA ATG TTA TCG ACT ATC AGA ATA GTG CCT TCC TCA GAT TGT TTC AGA CAG CAT GAA    598

 P   T   Y   S   F   F   V   I   Y   R   N   L   T   K   D   K   V   Y   N   E   L     176
CCG ACT TAC TCG TTT TTT GTG ATC TAC AGA AAT TTA ACA AAA GAC AAG GTG TAT AAC GAG TTA    661

 D   N   M   G   I   S   R   F   K   R   D   T   E   K   M   D   P   R   N   D   T     197
GAC AAT ATG GGG ATT TCT AGA TTC AAA CGA GAT ACG GAA AAG ATG GAT CCC AGA AAT GAC ACG    724

 C   N   V   V   P   W   T   V   D   F   A   E   L   G   W   D   K   F   I   S   F     218
TGT AAC GTT GTT CCA TGG ACT GTC GAT TTT GCC GAA CTG GGG TGG GAT AAG TTC ATT TCG TTT    787

 P   L   T   Y   N   A   N   A   C   E   G   K   C   A   Q   S   R   N   C   V   S     239
CCA CTT ACC TAT AAC GCA AAC GCA TGT GAA GGA AAA TGT GCA CAA TCT AGA AAC TGC GTC AGC    850

 A   M   N   K   P   I   N   M   L   K   N   Y   E   I   L   R   R   L   Y   A   Y     260
GCT ATG AAC AAG CCT ATC AAT ATG CTA AAA AAC TAC GAA ATT TTG CGA AGA TTA TAC GCG TAT    913

 C   P   D   V   S   L   L   D   H   S   C   C   R   P   S   K   Y   G   S   Q   G     281
TGT CCG GAT GTC TCT CTG CTG GAT CAC AGT TGT TGT AGA CCA TCC AAG TAT GGC TCA CAA GGC    976

 I   V   F   R   G   A   S   G   S   L   F   F   I   V   V   S   Q   M   R   V   L     302
ATT GTG TTT CGT GGC GCA TCT GGC TCT TTG TTT TTT ATA GTT GTT TCC CAG ATG CGA GTT CTT   1039

 E   C   S   S   N   *                                                                 308
GAG TGC AGC TCA AAT TGA  actgcttcgtcgagtttttttttatttgctaaaatcaatgtttcgcggcgatccttatc  1116

atccgtcctgttttcgatgtctcacattagaagtgtgttatgatttacattgatagattttgtcatctcacattaatatttatg  1200

atttttcattcatttaatcatcgtttttaaaacgttcgtgtattaaatacttcttacattgtttcattttaatccttgcttcta  1284

accactgca                                                                             1293
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Figure 2

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og-tgfb-like-Cg CNVVPWTVDFAELGWDKFISFPLTYNANACEGKCAQSRNCVSAMN----KPINMLKNYEI
BMP15-Ss        CSLHPFQVSFHQLGWDHWIIAPHFYTPNYCKGVCP--RVLHYGLN------SPNHA---I
BMP3-Hs         CARRYLKVDFADIGWSEWIISPKSFDAYYCSGACQ--FPMPKSLK------PSNHA---T
activin-Dm      CCKESFYVSFKALGWDDWIIAPRGYFANYCRGDCT--GSFRTPDTFQT-FHAHFIE---E
inhibin-Dr      CCKRQFYVNFKDIGWNDWIIAPSGYHANYCEGDCA--SNVASITGNSLSFHSTVIS---H
TGFb-Cg         CCRFTTTIAFSDLGWNDWILAPPDYEAHYCDGSCP-----DRFKM------ANTFA---G
BMP7-Xt         CKKHELYVSFKDLGWQDWIIAPEGYAAFYCEGECA--FPLNSYMN------ATNHA---I
BMP5/8-Hv       CDKHPLYIGFKDLGWSDWIIAPDGYRANYCGGDCS--FPLDNNAN------ATNHA---I
GDF9-dr         CDLYDFRVSFKELKLDHWIIEPKKYNPRYCKGSCP--RNVGFMYG------SPMHT---M
BMP5-Dr         CKKHELYVSFRDLGWQDWIIAPEGYAAFYCDGECS--FPLNAHMN------ATNHA---I
BMP2/4-Pm       CARYPLYVDFSDVGWNDWIVAPPGYNAFFCQGECH--FPLPQHLN------STNHA---I
AMH-Hs          CALRELSVDLRAERSVLI---PETYQANNCQGVCG--WPQSDRNP------RYGNH---V
BMP4-Xt         CRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCP--FPLADHLN------STNHA---I
BMP2-Pf         CKRKPLYVDFKAVGWNDWIFAPPGYEAYYCHGSCN--WPYDDHMN------VTNHA---I
60A-Dm          CQMQTLYIDFKDLGWHDWIIAPEGYGAFYCSGECN--FPLNAHMN------ATNHA---I
GSDF-Oc         CCPLASQIFLKDLGWENWVIYPESFTYVQCS-PCKS--RLDLSPSR----CPSHAPPAQD
                *                            *   *

og-tgfb-like-Cg LRRLYAYCPDVSLLDHSCCR-PSKYGSQGIVFRGASGSLFFIVVSQMRVLECSSN----
BMP15-Ss        IQNLVNELV QSV-PQPSCV-PYKYVPISILLIEANGSILYKEYEDMIAQPCTCR----32%
BMP3-Hs         IQSIVRAVGVVPGIPEPCCV-PEKMSSLSILFFDENKNVVLKVYPNMTVESCACR----28%
activin-Dm      YRKMGLM----NG-MRPCCA-PIKFSSMSLIYYGDDGIIKRDLPKMVVDE-CGCP----27%
inhibin-Dr      YRIRGYSPFT-NI--KSCCV-PTRLRAMSMLYYNEEQKIVKKDIQNMIVEECGCS----26%
TGFb-Cg         IQARLHALYPNKF-PKPCCV-PSKLSPLTILHKDSSGKYQLTDYPDMIVEDCKCA----26%
BMP7-Xt         VQTLVHFINPDTV-PKPCCA-PTQLNPISVLYFDDSSNVILKKYRNMVVRACGCH----25%
BMP5/8-Hv       IQTLVHMMYPEII-PKPCCA-PNKLNTLQVLFLDERNNVVMKRYSNMIVQHCGCQ----25%
GDF9-dr         VQNLIYEKLDSSV-PRPTCV-PSEYNPLSVLTFENDKSYAYKEYEEMIATKCACR----25%
BMP5-Dr         VQTLVHLMFPDNV-PKPCCA-PTKLNAISVLYFDDSSNVILKKYRNMVVRSCGCH----24%
BMP2/4-Pm       VQTLVNSVNP-EV-PRACCI-PTELTPIALLYLDEYEKVVLKNYQDMVVEGCGCR----23%
AMH-Hs          VLLLKMQARGAALARPPCC-VPTAYAGKLLISLSEERISAHHVPNMV-ATECGCR----23%
BMP4-Xt         VQTLVNSVNS-SI-PKACCV-PTDLSAISMLYLDEYDKVVLKNYQEMVVEGCGCR----23%
BMP2-Pf         VQDLVNSINPGSV-PKPCCV-PTELSSLSLLYTDEHEVVVLKVYPDMVVEGCGCR----23%
60A-Dm          VQTLVHLLEPKKV-PKPCCA-PTRLGALPVLYHLNDENVNLKKYRNMIVKSCGCH----22%
GSDF-Oc         TPSQM-----------PCCQ-TTSTEHVPFLYM--DEFSTLTIPSVQLTRACGPGN---12%
                                 **                                *  *
```

Figure 3

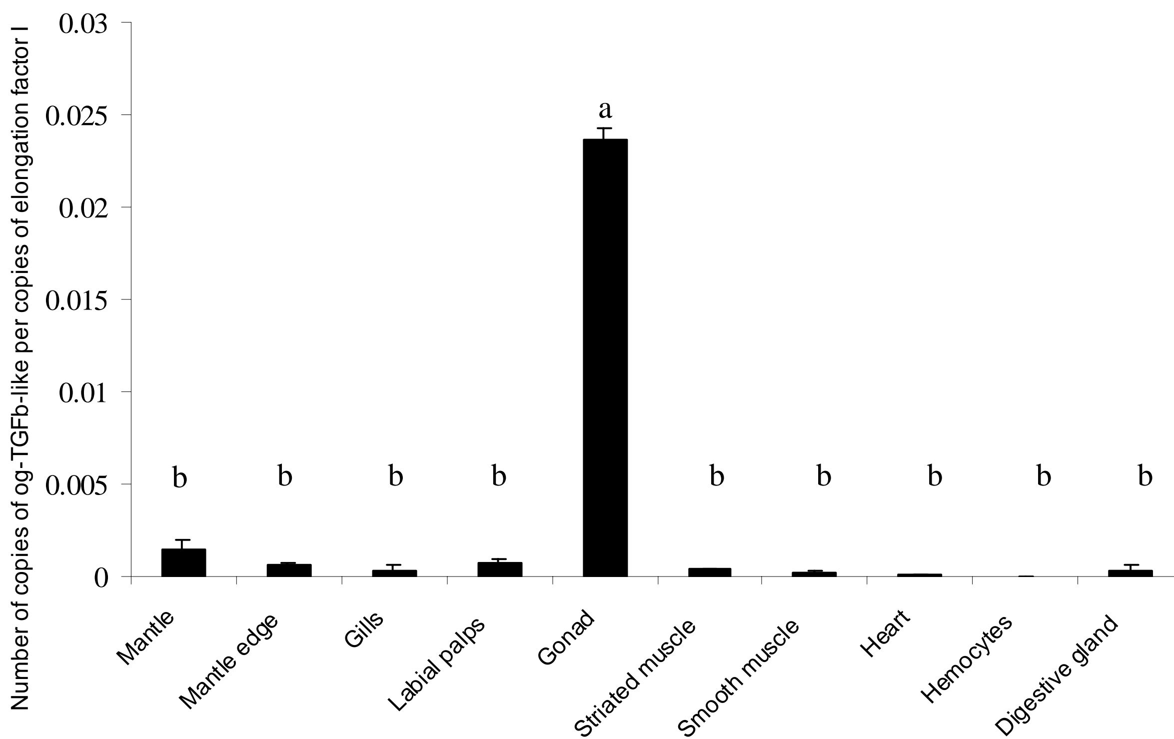

Figure 4

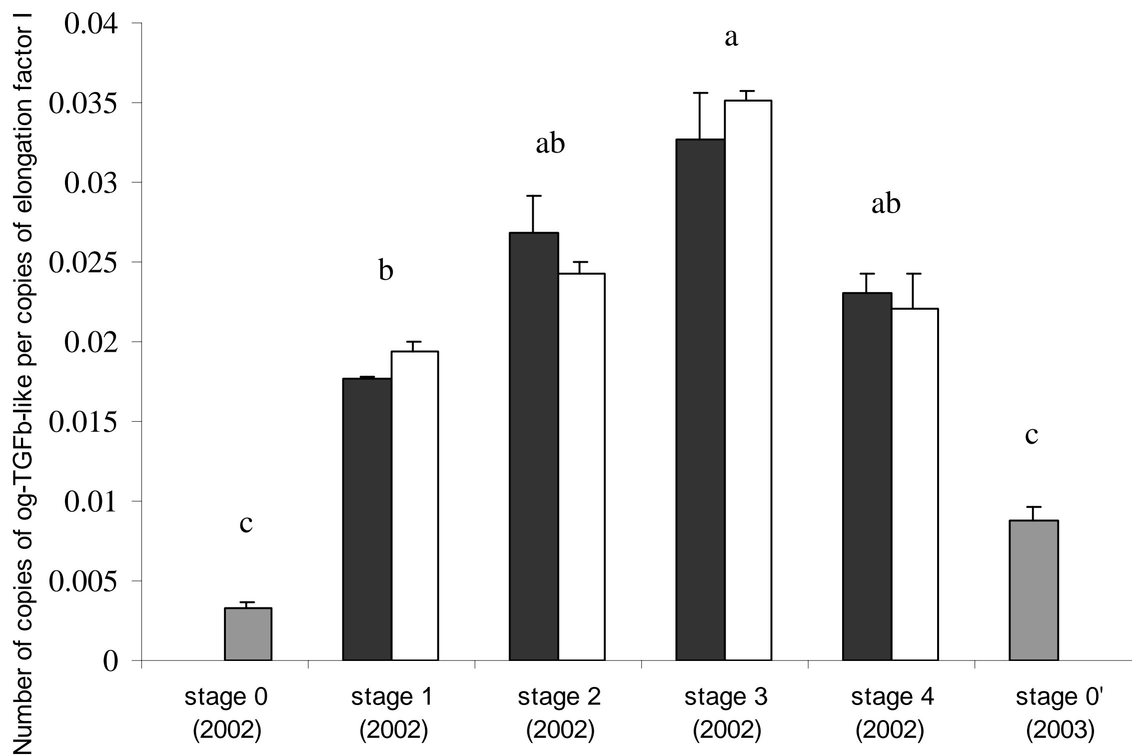

Figure 5

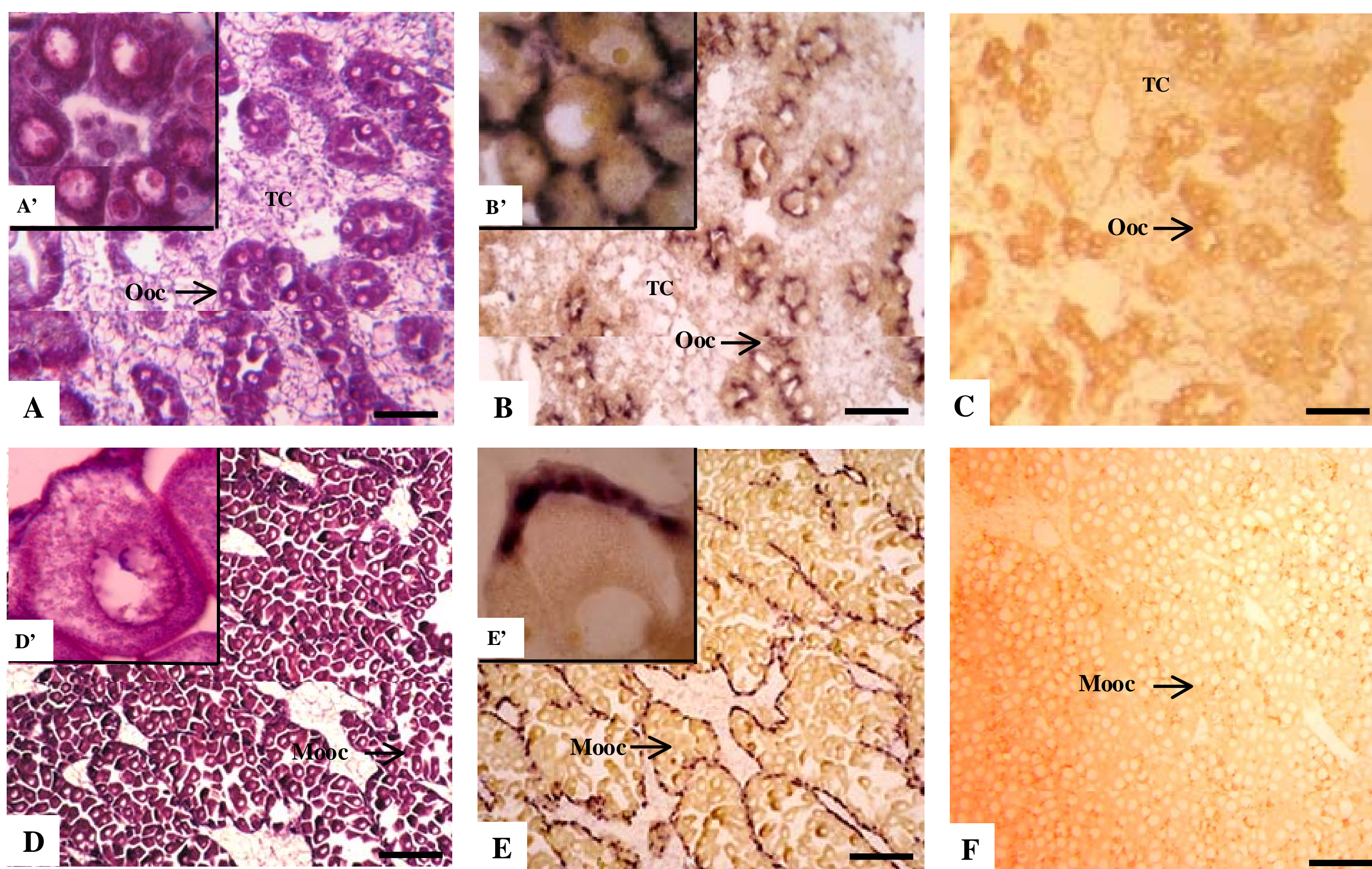

Figure 6