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ORIGINAL ARTICLE

DNA Methylation Is Crucial for the Early Development in the Oyster *C. gigas*

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Abstract In vertebrates, epigenetic modifications influence gene transcription, and an appropriate DNA methylation is critical in development. Indeed, a precise temporal and spatial pattern of early gene expression is mandatory for a normal embryogenesis. However, such a regulation and its underlying mechanisms remain poorly understood in more distant organisms such as Lophotrochozoa. Thus, despite DNA in the oyster genome being methylated, the role of DNA methylation in development is unknown. To clarify this point, oyster genomic DNA was examined during early embryogenesis and found differentially methylated. Reverse transcriptase quantitative polymerase chain reaction indicated stage-specific levels of transcripts encoding DNA-methyltransferase (DNMT) and methyl-binding domain proteins. In addition, as highlighted by electronic microscopy and immunohistochemistry, the DNMT inhibitor 5-aza-cytidine induced alterations in the quantity and the localisation of methylated DNA and severe dose-dependent development alterations and was lethal after zygotic genome reinitiation. Furthermore, methyl-DNA-immunoprecipitation–quantitative polymerase chain reaction revealed that the transcription level of most of the *homeobox* gene orthologues examined, but not of the other early genes investigated, was inversely correlated with their specific DNA methylation. Altogether, our results demonstrate that DNA methylation influences gene expression in *Crassostrea gigas* and is critical for oyster development, possibly by specifically controlling the transcription level of *homeobox* orthologues. These findings provide evidence for the importance of epigenetic regulation of development in Lophotrochozoans and bring new insights into the early life of *C. gigas*, one of the most important aquaculture resources worldwide.

Keywords Epigenetics · Development · Methylation · Homeobox · Evolution

Abbreviations

5-aza-C	5-Aza-cytidine
5-mC-IPed	5-Methylcytosine immunoprecipitated
DNMT	DNA methyl-transferase
hpf	Hours post-fertilization
MBD	Methyl-binding domain
MeDIP	Methyl-DNA immunoprecipitation
PRC	Polycomb repressive complex

Introduction

Among the pathways responsible for gene expression regulation, epigenetic mechanisms appear important in stabilising the probabilistic events governing gene expression into transient,

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dynamically stabilised transcriptomes (Mas et al. 2011; Meissner 2010). Indeed, it has now been widely demonstrated that histone chemical modifications can influence the compaction of chromatin and therefore the transcriptional activity within the concerned genomic regions (Cervera et al. 2009; Lan et al. 2007). DNA methylation also exerts such an influence, either by recruiting chromatin modifying enzymes or by interfering with the DNA binding of the transcription machinery in a direct fashion or via methyl-DNA binding domain (MBD) proteins (Lindeman et al. 2010; Liang et al. 2011). In vertebrates, the methylation of regulatory regions has been demonstrated as an important regulator of mRNA expression for many different genes (Rivière et al. 2011; Yang et al. 2011) and noticeably for *homeobox* genes in the context of embryonic development (Branciamore et al. 2010; Hershko et al. 2003; Laurent et al. 2010; Liang et al. 2011). This regulatory mechanism has been described from fishes to mammals and therefore is hypothesised to be generally conserved in vertebrates. DNA within coding sequences can also be methylated, though the relationship between the amount and/or position of methylcytosines and mRNA expression level is less clear. Indeed, gene body methylation was found positively correlated with transcription of moderately expressed genes in insects (Xiang et al. 2010) and plants (Zemach et al. 2010). In contrast, genes that exhibit either very high or very low transcriptional activity were found less methylated, such as in rice (Zemach et al. 2010). In Ecdysozoa like *Drosophila* or *Caenorhabditis elegans*, genomic DNA is poorly methylated, (for review, see (Bird 2002)). Yet, recent studies highlight the role of DNA methylation in the early life of the jewel wasp *Nasonia* (Zwier et al. 2012). However, the putative implication of this epigenetic feature in the development of more distant species remains extremely elusive, despite DNA being found methylated in a broad range of organisms throughout animal evolution (Regev et al. 1998).

The specific and precise mechanisms stabilising the stochastic expression of developmental genes are critical for a proper embryogenesis (Laforge et al. 2005; Raj and van Oudenaarden 2008). Failure in the establishment of their precise temporal and spatial expression patterns may lead to abnormal body axes and/or incomplete structures within embryos and eventually be lethal. In this context, the genes belonging to the *Hox* family, i.e. which bear a homeodomain or a homeobox coding sequence and which are clustered within defined loci, are crucial regulators of early development. The specific distribution of such transcription factors (for review, see (Pearson et al. 2005)), which are able to diffuse between cells across the embryo (Layalle et al. 2011; Di Lullo et al. 2011), regulate the downstream expression of a great number of genes. These regulatory cascades lead to the cell lineage commitment and differentiation by defining positional identities and stabilising cell-specific transcriptomes. Interestingly, the general role and critical implications of *homeobox* genes are evolutionary conserved, even though slight differences in their precise functions may exist between distant animal groups. For example, while the *Orthopedia* gene is expressed during the development of the central nervous system in mice (Simeone et al. 1994), *Drosophila* (Simeone et al. 1994) and *Patella* (Nederbragt et al. 2002), its orthologue in the sea urchin (Di Bernardo et al. 1999) is implicated in the establishment of the skeleton. Different studies have demonstrated that *homeobox* genes are genetically conserved in mollusks (Barucca et al. 2003; Lee et al. 2003; Canapa et al. 2005; Pérez-Parallé et al. 2005; Mesías-Gansbiller et al. 2012; Zhang et al. 2012), even though it remains unclear whether their precise function as well as the rule of colinearity holds true for every group (Frobius et al. 2008; Zhang et al. 2012).

The oyster *Crassostrea gigas* is a bivalve mollusk which belongs to the Lophotrochozoa, a distant and largely underdescribed group in spite of being the sister clade of Ecdysozoa among protostomes. Because oysters constitute one of the most important aquaculture resources worldwide, it is emerging as a well-studied species and extensive genomic data have become available (Fleury et al. 2009; Zhang et al. 2012). Besides, the presence of DNA methylation within the oyster genome was recently demonstrated (Gavery and Roberts 2010). It is hypothesised to participate in phenotypic plasticity leading to both short- and long-term adaptation to environmental inputs through increased transcriptional regulatory opportunities and mutation probability, respectively (Roberts and Gavery 2012). Because oysters are estuarine animals, they are especially subjected to changing environmental cues. This raises the question of whether DNA methylation has functional implications on gene expression and especially whether such a DNA modification plays a role in the regulation of early development in *C. gigas*.

To gain more insights into these issues, we examined the CpG methylation in the oyster genomic DNA along early development, i.e. from oocytes to the D-larvae stage using fluorescent ELISAs. Then, using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), we examined whether oyster DNA methyltransferases (DNMTs) and MBD orthologues are expressed and, if so, display differential expression levels during embryogenesis and larval development. In addition, the influence of the DNMT inhibitor 5-aza-cytidine (5-aza-C) was investigated in vivo using immunohistochemistry and scanning electronic microscopy. Furthermore, the mRNA expression level of several early genes, including *homeobox*, polycomb-repressive complex and putative epigenetic regulators orthologues, was characterised using RT-qPCR. In parallel, these transcript levels were investigated for susceptibility of gene-specific regulation by DNA methylation by methylated-DNA immunoprecipitation (MeDIP)-qPCR.

Materials and Methods

Animals

Adult 2-year-old *C. gigas* (Thunberg 1793) specimens were purchased from an oyster farm (Blainville, Manche, France). Embryos, larvae and spat were obtained at the IFREMER experimental hatchery (Argenton, France). Reproductive stage and sex were histologically determined as follows: stage 0 (sexual resting stage), male and female stage I (gonial multiplication stage), stage II (gametes maturation) and stage III (sexual maturity) (Berthelin et al. 2000). Diploïdy of oysters was assessed by propidium iodide flow cytometry on gill cells of randomly sampled animals as previously described (Jouaux et al. 2010).

Fertlisation Assays and Early Development in the Presence of a DNMT Inhibitor

Broodstock *C. gigas* specimens were purchased from an oyster farm in Guernsey (Guernsey, GB) or obtained in the IFREMER experimental hatchery (Argenton, France). Gonads were scarified, and gametes were filtered on a 100 μm mesh for the removal of large debris. For females, oocytes (oo) were harvested as the remaining fraction on a 30 μm mesh; for males, spermatozoa (spz) were harvested as the passing fraction on a 30 μm mesh. Oocytes were pre-incubated in filtered-sterile (0.22 μm) seawater (FSW) alone (Controls) or in the presence of 10^{-7}, 10^{-6} or 10^{-5} mol L^{-1} of the DNMT inhibitor 5-aza-cytidine (Sigma A2385, France) at 25 °C for 15 min. The drug was diluted immediately before use in FSW at 10^{-5} mol L^{-1} and then further diluted in FSW when needed. Fertilisations were triggered by the addition of spermatozoa and were carried out in oxygenated FSW at 25 °C (500 oo L^{-1}; ca. 100 spz/oo). Embryos were left unattended until sampling, i.e. before fertilisation for control oocytes, and ca. 1 h post-fertilisation (hpf) for two to four cell stage, ca. 3 hpf for morulae, ca. 6 hpf for blastulae, ca. 9 hpf for gastrulae, ca. 16 hpf for trochophore larvae and ca. 24 hpf for D larvae. Development stages were assayed by microscopic observation based on morphological and motility criteria before and after fixation using 70 % ethanol. Embryos that could not be assigned a development stage based on the former criteria were considered 'abnormal'. The development of 5-aza-cytidine–treated embryos was monitored in parallel using the same method. Animals from the 10^{-5} mol L^{-1} experiment were harvested after 6 (5-aza-6 h) and 24 h (5-aza-24 h), respectively. Samples were split in aliquots, stored dry (for DNA extraction) or in Tri-reagent (Sigma T9424, France) (for RNA extraction) at −80 °C and thawed only once before use.

DNA Methylation Quantification

Genomic DNA from controls, 5-aza-6 h and 5-aza-24 h embryos was purified using affinity chromatography (Macherey Nagel 740952, Germany). Then, purified genomic DNA (100 ng) was submitted to 5-methylcytosine (5-mC) fluorimetric ELISA using the Methylflash DNA Fluorimetric Quantification Kit according to the manufacturer's instructions (Epigentek P-1035, New Jersey, USA). Briefly, samples were incubated in the presence of a 5-methylcytosine antibody coated on a multiwell plate. After binding (90 min, 37 °C) and extensive wash, samples were incubated with a secondary antibody. The binding of this antibody was then quantified by the addition of a fluorogenic substrate, and fluorescence was measured (Berthold Mithras 940LE; excitation, 530 nm, emission 590 nm). The absolute amounts of 5-mC in the samples were resolved versus a 5-mC standard curve established in parallel in the same assay.

Embryo Whole Mount Immunohistochemistry

Samples were fixed in MEM-PFA-T and stored at 4 °C until use. They were then recovered by centrifugation (220×*g*, 10 min), resuspended in 2 N hydrochloride, incubated for 30 min at 37 °C and decanted. Embryos were resuspended in blocking buffer (PBT, 10 % BSA, room temperature for 2 h). Blocking buffer was removed, and embryos were incubated with the primary antibody (Sheep anti 5Me-C, Epigentek A1014 (New Jersey, USA), $1/100^{th}$ dilution in PBT/5 % BSA) overnight at 4 °C. Samples were washed in blocking buffer then incubated with the secondary antibody (Anti sheep-FITC, SantaCruz K1408 (California, USA), $1/200^{th}$ dilution in PBT/2.5 % BSA) for 1 h at room temperature. Samples were washed in PBT, mounted on slides and visualised under an epifluorescence microscope (Nikon Eclipse 80i).

Scanning Electron Microscopy

The samples were fixed with 3.2 % glutaraldehyde in 0.31 mol L^{-1} cacodylate buffer pH 7.4 in presence of 0.25 mol L^{-1} sucrose at 4 °C during 90 min. The cells were rinsed three times in cacodylate buffer 0.4 mol L^{-1} pH 7.4 in presence of 0.3 mol L^{-1} sucrose. The samples were sedimented on Thermanox® coverslips coated with poly-L-lysine during several days. The samples were then post-fixed with 1 % osmium tetroxyde in 0.2 mol L^{-1} cacodylate buffer pH 7.4 in presence of 0.36 mol L^{-1} sucrose (1 h, 4 °C protected from light). The larvae were rinsed in 0.4 mol L^{-1} cacodylate buffer pH 7.4 in presence of 0.3 mol L^{-1} sucrose, then dehydrated in increasing ethanol concentrations (70–100 %) and finally critical point dryed (CPD 030 LEICA Microsystem). The cells were sputtered with platinum and observed with a scanning electron microscope JEOL 6400 F.

In Silico Analyses

The *C. gigas* EST database (Fleury et al. 2009) version 7 (Gigasdatabase v.7) was screened for annotations of putatively

conserved *homeobox* genes (*Abd*, *Alx-4*, *Eyegone*, *Hmtx*, *Hoxpost*, *Mox2*, *Notochord*, *Orthopedia*, *Post2*, *Prospero*). These genes were chosen regarding their high sequence conservation with *homeobox* genes throughout metazoan animals, thereby displaying putative functional conservation in early developmental processes in the oyster. The putative *Drosophila* and mammalian orthologues of these oyster genes are located 3' to 5' of the *Hox* cluster, thereby displaying gradual temporal expression along development and relative antero-posterior identity. The selected polycomb repressive complex (PRC) genes (*BMI-1*, *EED*, *PHC*, *Sfmbt*, *Suz12*) have their orthologue demonstrated or suspected to mediate development genes silencing through epigenetic regulation (Terranova et al. 2006; Bantignies and Cavalli 2006). The orthologues of the examined epigenetic regulators are implicated in a wide range of epigenetic processes (DNA methylation machinery: *DNMT1*, *DNMT2*, *DNMT3b*, *CXXC-1*, *MBD2*, *MeCP2*; Histone modifiers: *JmjCA*, *JmjCB*, *JmjD6*, *KDM2*, *OSA*) (Table 1). In the case where no annotation of the aforementioned genes was available, putative corresponding ESTs were searched by screening the Gigasdatabase with the corresponding mammalian and/or *Drosophila melanogaster* and/or *C. elegans* protein sequences using the TBLASTN online tool (http://blast.ncbi.nlm.nih.gov) with default parameters. Similarity was considered significant at *e*-value$<10^{-30}$. Further analysis on the oyster genome (Zhang et al. 2012) indicated that only the examined DNMT1, DNMT2 and DNMT3 orthologues would be able to methylate cytosines. The EST numbers, target sequence position within EST and corresponding scaffold, predicted CDS, coding strand, accession number, number of exons in corresponding predicted genes and position of target sequences within are summarised in Table 1. A CpG island analysis is also provided for each examined gene, as given by the CpG plot program (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot). A sequence ranging from 3 kb upstream the putative translation start site and encompassing the whole predicted CDS was analysed using the following parameters: length, 100; window, 100; %C+G, >45; CpG O/E, >0,6 (Gardiner-Garden and Frommer 1987; Rice et al. 2000).

RT-qPCR

Total mRNA was isolated from control and 5-aza-cytidine-treated oocytes, embryos and larvae (~200,000 animals per sample) as previously described (Riviere et al. 2011). Briefly, samples were extracted using Tri-Reagent (Sigma T9424, France), then RNA were purified from the resulting aqueous phase using affinity chromatography (Nucleospin RNA II kit, Macherey-Nagel 740955, Germany). RNA concentrations were assayed using a Nanodrop 1000 spectrophotometer (ThermoScientific, France). After digestion with 1 U RQ1 DNAse (Promega M6101, France) for 30 min to prevent genomic DNA contamination, 250 ng of total RNA were reverse-transcribed using 200 U of M-MLV RT (Promega M1705, France) and 100 ng of random hexamers. Resulting cDNAs were diluted, and the equivalent amount of 5 ng of starting RNA was assayed for target gene expression using the elongation-factor alpha (EFα) (GenBank accession number BAD15289) transcript as reference gene. SYBR-green quantitative PCR was performed on a CFX96© apparatus (Bio-Rad, France). Gotaq qPCR master mix (Promega A6001, France) was used in 40 cycles (95 °C/15 s, 60 °C/15 s) reactions with the target gene (see above and Table 1) or EFα (Qs-*Cg*-EF: 5′-ACCACCCTGGTGAGATCAAG-3′ and Qa-*Cg*-EF: 5′-ACGACGATCGCATTTCTCTT-3′) primers respectively. A standard curve consisting in a dilution series of the cDNA library plasmid was used to check for qPCR efficiency for each primer pair. All primer pairs used herein were checked for 90 %$<E<$110 %, where *E* represents the PCR efficiency (see Supplementary table for primer sequences). Accurate amplification of the target amplicon was checked by performing a melting curve and an end-point agarose gel electrophoresis followed by ethidium bromide staining. A parallel amplification of the reference gene (EFα) (Ref gene) was carried out to normalise the expression data of the target gene transcript. The relative level of target gene expression was calculated for one copy of the reference gene by using the following formula, $N=2^{(\text{Ct Ref gene} - \text{Ct target gene})}$. The same formula was used to estimate the EFα mRNA content in the control versus the 5-aza-C groups, except that the mean expression value of all the other genes at the same stage was considered as the reference. Water was used instead of cDNA as a blank for amplification, and DNAse-untreated cDNA was used as a negative control to check for absence of genomic DNA contamination. All samples were analysed at least in triplicate to establish the mRNA expression profile of the target genes.

Methyl-DNA Immunoprecipitation (MeDIP)

The immunoprecipitation of methylated DNA was carried out using the EpiQuick Tissue Methylated DNA Immunoprecipitation Kit (Epigentek P-2020, New Jersey, USA) following the manufacturer's instructions. Briefly, up to 20 mg of sample (oocyte, embryo or larvae) were disaggregated and centrifuged (220×*g*, 5 min, 4 °C). Supernatant was removed, and sample DNA was sheared by sonication on ice until the size of most DNA fragments (as resolved using ethidium bromide agarose gel electrophoresis) ranged between 300 and 600 bp (16 pulses of 10 s at 40 % power on a Bioblock Scientific VibraCell 72442 probe sonicator, with 30-s rest intervals on ice between pulses), then centrifuged (5,000×*g*, 10 min, 4 °C) for removal of cell debris. Supernatants were then recovered, diluted in ChIP dilution buffer, and an aliquot was removed ('input DNA'). Samples were then immunoprecipitated using a 5-methylcytosine antibody coated on a multiwell plate and

Table 1 Overview of the examined genes and corresponding target sequences

Gene name	Alternative annotation	EST	Target sequence (cf. EST)	Oyster genome scaffold	CDS		Strand	Oyster DB accession (OYG_100…)	CpG Islands (length, start pos.)	Nb. of exons in predicted gene	Target sequence (cf. CDS)
					Start	Stop					
Abd	Abd-A/Ubx/Lox2	CU994613	131–250	247	139,499	148,449	(+)	18592	186, −1,251 165, −57 287, +109	2	Exon 1 (+63)
Alx-4	Lhx5	AM566498	82–179	206	150,258	170,090	(+)	25343	148, −713 105, −387 132, +64	6	5'UTR (−452)
BMI-1		CU991431	468–574	1,887	43,003	37,272	(−)	06622	143, −2,516 157, +3,360 211, +5,125 171, +5,504	8	Exons 5–6 (+4,447)
CXXC-1		FP007125	1,803–1,912	1,852	24,273	33,277	(−)	03574	101, +26 211, +5,781	14	Intron 1 (+154)
DNMT1		CU99437	146–250	132, 1,862, 41,550	35,280 618,220 63,903	32,606 620,050 62,676	(−) (+) (−)	N/A 21920 N/A	N/A	N/A	N/A
DNMT2	TRMT	CU997720	4–101	1,736	5,312	824	(−)	20946	106, −2,506 156, −1,736	9	Exon 6 (+3,164)
DNMT3b	DNMT3a	CU989755	20–126	1,763	443,571	430,939	(−)	19546	104, −1,389	21	Exon 21 (+11,574)
EED	Extra Sex Combs	AM862109	561–650	161	14,324	26,281	(+)	08582	185, +1,381 107, +3,535 117, +6,319 281, +6,913	12	Intron 5 (+4,453)
Eyegone	Pax6	CU990067	252–359	361	614,882	611,493	(−)	20873	247, −2,148	4	Exon 3 (+2,561)
Hmtx	Meis1	AM863045	295–403	1,794	383,732	365,293	(−)	19589	119, +12,991	6	Exons 4–5 (+6,346)
Hoxpost	HoxC11/Post1	CU987796	187–288	1,179	702,063	687,667	(−)		170, −725 114, −441 136, −23 245, +189		Exon 1 (+59)
								27385	121, +5,773	2	
								27386	130, +5,992 103, +6,585 186, +7,113 152, +8,443 142, +11,680 164, +14,087	2	
JmjCA	C2orf60	CU986798	484–589	1,121	101,942	92,961	(−)	06502	113, −2,072 103, −1,010 105, −69 188, +550	9	Exon 6 (+8,957)

Table 1 (continued)

Gene name	Alternative annotation	EST	Target sequence (cf. EST)	Oyster genome scaffold	CDS		Strand	Oyster DB accession (OYG_100…)	CpG Islands (length, start pos.)	Nb. of exons in predicted gene	Target sequence (cf. CDS)
					Start	Stop					
									100, +2,891		
									207, +8,716		
JmjCB	JmjD4	HS165887	409–500	514	348,665	346,264	(−)	18360	N/A	6	5'UTR-Exon 1 (−10)
JmjD6		CU997249	282–378	160	318,016	312,810	(−)	20587	N/A	7	Exon 5 (+3,167)
KDM2		EW779134	102–214	454	293,393	275,736	(−)	11640	120, −1,791	8	Exon 4 (+1,281)
									117, +9,360		
									115, +11,443		
									129, +11,857		
MBD2		CU988032	238–334	787	251,947	273,262	(+)	11651	159, −75	6	Exons 2–3 (+5,201)
									115, +9,236		
									117, +9,529		
									115, +15,704		
									113, +19,333		
MeCP2		FP004144	26–121	1,605	617,547	606,395	(−)	23379	120, −2,924	6	Intron 1 (+6,195)
									160, +8,500		
Mox2	Meox2	CU992037	544–637	1,722	316,246	308,195	(−)	14888	139, −2,634	3	Exon 2 (+7,005)
									151, −2,052		
									117, −916		
									130, −295		
									117, +47		
									128, +169		
									255, +7,153		
Notochord	Not/HoxB1	CU998426	258–370	960	4,704	778	(−)	13404	201, +14	3	Exon 1 (+13)
									111, +256		
									106, +775		
Orthopedia	Otp	CU991046	145–248	343	342,976	345,715	(−)	21751	113, −2,286	3	Exon 3 (+5,381)
									107, −240		
									268, +843		
									157, +1,687		
									180, +3,205		
									457, +5,291		
OSA	ARID1A	CU991972	170–259	42,486	132,151	132,222	(+)	N/A	N/A	N/A	N/A
PHC	PHC-2	EW778754	1,053–1,159	43,426	14,977	1,665	(−)	2690	N/A	15	Exons 13–14 (+11,768)
				41,754	128,021	127,600	(−)	5584		5	Exons 3–4 (+450)
Post2	Abd-B	AM857383	229–329	1,179	824,809	830,848	(+)	27388	122, −2,950	2	Exon 1 (+341)
									344, +155		
									117, +1,129		
									104, +1,612		
									173, +3,303		

Table 1 (continued)

Gene name	Alternative annotation	EST	Target sequence (cf. EST)	Oyster genome scaffold	CDS		Strand	Oyster DB accession (OYG_100…)	CpG Islands (length, start pos.)	Nb. of exons in predicted gene	Target sequence (cf. CDS)
					Start	Stop					
									101, +5,265		
Prospero	Prox1	CU685165	30–128	106	157,153	147,101	(−)	22026	100, −2,915 103, −1,807 129, −1,310 127, +2,797 133, +7,368 132, +9,654	5	5'UTR (−4,243)
Sfmbt		FP006430	18–121	1,723	125,458	118,398	(−)	10519	135, +6,630	19	Exon 15 (+4,563)
Suz12		AM863465	221–326	619	212,714	220,270	(+)	25759	183, −110 133, +5,019	13	Exons 10–11 (+5,583)

incubated on an orbital shaker (2 h, 100 rpm, room temperature). After extensive washing, samples and input DNA were released from the antibody and recovered by proteinase K treatment (15 min, 65 °C). DNA (samples and inputs) was then purified using affinity chromatography. For downstream experiments, 35 ng of immunoprecipitated or input DNA were subjected to parallel qPCR reactions using validated specific primer pairs of the selected genes, as described above. The gene-specific DNA methylation was defined, in this study, as the ratio of the qPCR signal obtained for DNA after methyl-CpG-immunoprecipitation and the qPCR signal obtained for the input DNA for the same specific gene and given as 'IP/Input' using the following formula, $\text{IP/Input} = 2^{(\text{Ct input} - \text{Ct IP})}$.

Statistical Analysis

All the results are given as the mean±SEM (standard error to the mean) of at least triplicate experiments. They were analysed for statistical significance using one-way (DNA methylation during development, mRNA expression during development, gene-specific methylation profiles), or two-way ANOVA (influence of 5-aza-C on gene expression at 6 and 24 hpf,) followed by Bonferroni's post hoc test. Chi square (influence of 5-aza-C on oyster development) and two-tailed Student's *t* test with Welch's correction were used when required (influence of 5-aza-C on DNA methylation). Correlation was tested at 95 % confidence intervals using one-tailed Pearson's or Spearman's test when required (IP/input versus mRNA expression). $p<0.05$ was considered significant. Data were analysed, and graphs were plotted using the Graphpad Prism software version 5.0.

Results

The Methylation of Genomic DNA Changes During the Oyster Development

The global methylation of genomic DNA was not constant over the early development of oysters. Indeed, quite heavily methylated within oocytes, oyster DNA became significantly more methylated up to the morula stage and then DNA methylation decreased until the gastrula stage (Fig. 1a). However, DNA seemed more methylated within trochophore larvae than within gastrulae and D-larvae, although this variation was not statistically significant (Fig. 1a). Such a methylation looked homogenous and widespread all over the embryo, at least up to the trochophore stage (Fig. 1b). Apparent inconsistency between ELISA and IHC, which cannot be considered quantitative, is likely explained by the chromatin conformation within embryonic cells.

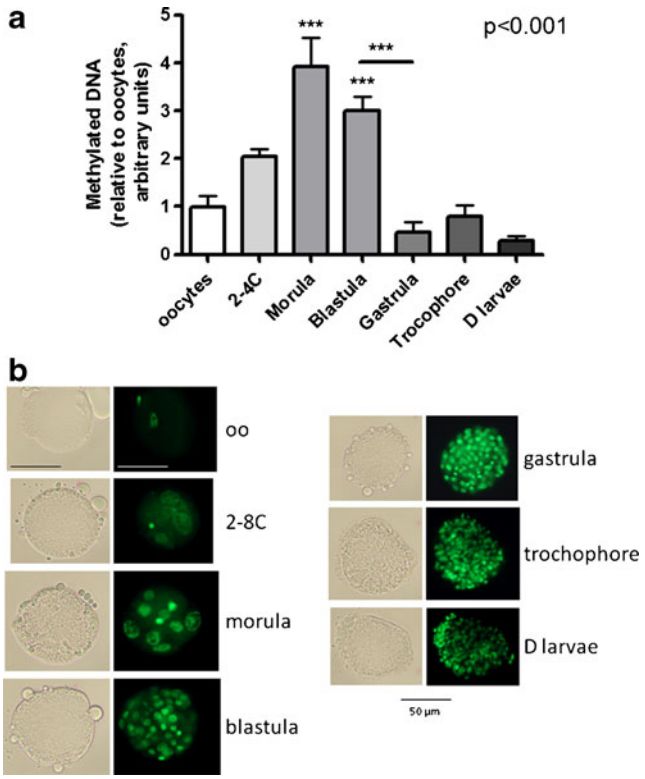

Fig. 1 CpG DNA methylation during early development in *C. gigas*. **a** Amount of CpG methylation during the early development stages using fluorescent ELISAs. The results were normalized regarding oocytes and are given for 100 ng genomic DNA; ***$p<0.001$, one-way ANOVA followed by Bonferroni's post hoc test; **b** localisation of methylated DNA within *C. gigas* embryos using a sheep anti-5-mC antibody and a FITC anti-sheep secondary antibody; *left*, transmitted bright field photonic microscopy; *right*, transmitted UV light epifluorescence microscopy using a 395–420 nm filter (FITC); the embryonic stage is indicated; oo, oocytes; 2–8C, 2–8 cells; magnification, ×400. *Nb*: bubbles at embryo edges are mounting artifacts. Scale *bar* 50 µm

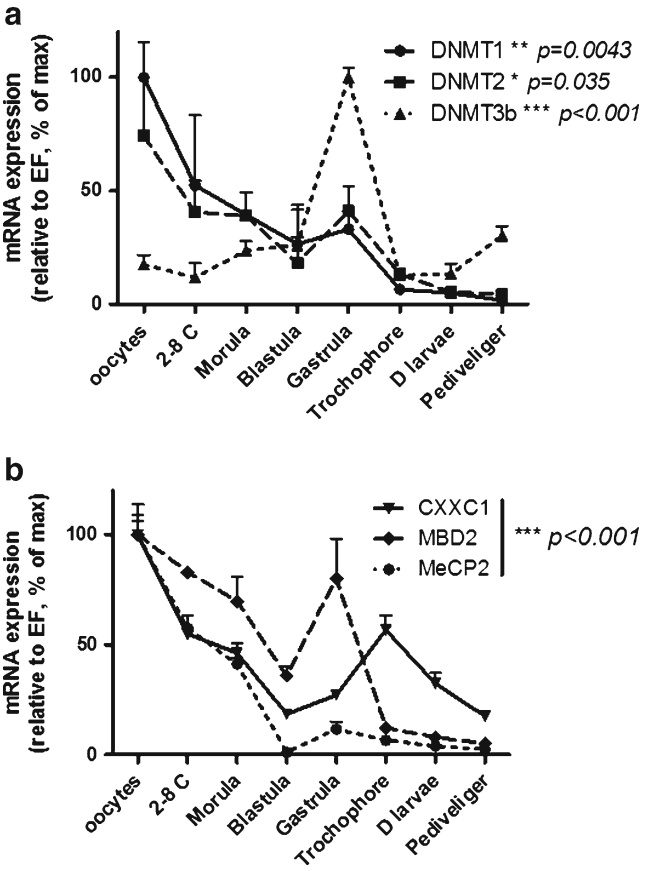

Fig. 2 mRNA expression level of DNMT (**a**) and MBD orthologues (**b**) during early development in the oyster. Expression levels were normalised regarding the elongation factor alpha (EFα) and are given relative to the maximum expression level (100 %) for each gene; *plus sign* $0.05<p<0.1$; *$p<0.05$; **$p<0.01$; ***$p<0.001$, one-way ANOVA followed by Bonferroni's post hoc test

Messenger RNA Expression Levels of DNMT and MBD Orthologues During Oyster Development

The transcript levels of the DNMT orthologues did not display the same profile during the oyster development. Indeed, *DNMT1* and *DNMT2* displayed maximum mRNA levels in oocytes and decreased till the pediveliger stage (Fig. 2a). In contrast, mRNA levels of *DNMT3b* were faintly detectable except in gastrulae. Interestingly, *DNMT1* and *DNMT2* transcripts were much more represented than *DNMT3b* transcripts, when compared with the elongation factor alpha. Indeed, the highest mRNA levels of *DNMT3b* (at the gastrula stage) reached ca. one tenth of the maximum *DNMT1* expression (in oocytes) (mRNA level relative to EFα: DNMT3b, $7.56\times10^{-4}\pm3.13\times10^{-5}$ (n=4) versus DNMT1, $1.03\times10^{-2}\pm3.62\times10^{-3}$ (n=6), respectively). The transcript levels of three identified methyl-DNA binding proteins ESTs also displayed variations during the early development in the oyster. Overall, *CXXC-1*, *MBD2* and *MeCP2* exhibited a biphasic decrease from oocytes to pediveliger larvae, with a peak at ca. 90 % of the maximum expression at the gastrula stage for *MBD2*, ca. 60 % at the trochophore stage for *CXXC-1* and ca. 15 % at the gastrula stage for *MeCP2* (Fig. 2b).

5-Aza-cytidine Treatment Decreases Genomic DNA Methylation

Treatment with 5-aza-cytidine induced a significant decrease (ca. 60 %) in DNA methylation at 6 and 24 h post-fertilisation (hpf) (Fig. 3a), indicating that treatment with a DNMT inhibitor induces an efficient DNA demethylation. This decrease was not always consistent between embryos, since the immunolocalisation signal of methylated DNA could be either homogenous or heterogeneous across the treated embryos at 6 hpf (Fig. 3b).

DNA Demethylation Induces Alterations of Oyster Development

The decrease in DNA methylation during oyster development induced a deleterious phenotype. Indeed, 5-aza-C-

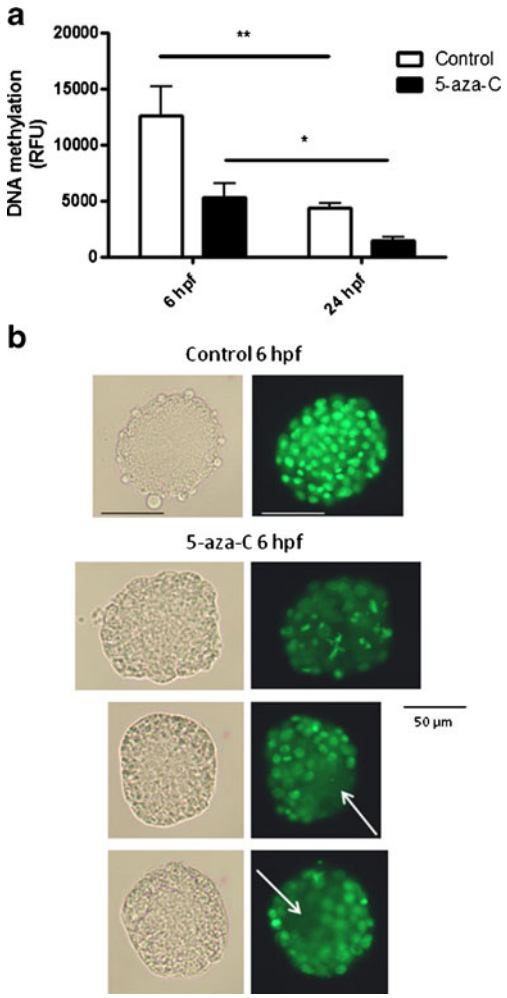

Fig. 3 Influence of 5-aza-C on CpG methylation. **a** Quantification of 5-mC within oyster genomic DNA at 6 and 24 hpf. *$p<0.05$; **$p<0.01$; ***$p<0.001$, two-way ANOVA followed by Bonferroni's post hoc test. **b** localisation of methylated cytosines within *C. gigas* embryos at 6 hpf in the presence (5-aza-C 6hpf) or the absence (control 6 hpf) of 10 µmol L^{-1} 5-aza-cytidine. *Arrows* indicate heterogeneity of DNA demethylation

treated animals displayed a significant dose-dependent alteration in embryogenesis from 6 hpf, i.e. after zygotic genome re-initiation but not before (χ^2 test of frequency distribution of developmental stages between control, 5-aza-C 10^{-7} mol L^{-1}, 5-aza-C 10^{-6} mol L^{-1}, 5-aza-C 10^{-5} mol L^{-1} samples, 1 hpf, p=0,715 n.s.; 3 hpf, p=0.675 n.s.; 6 hpf, $p<0.0001$***; 24 hpf, $p<0.0001$***). At 24 hpf, ca. 75 % of the control embryos were D-larvae, whereas less than 10 % of the oysters treated with 10^{-5} mol L^{-1} 5-aza-C could reach the trochophore stage (Supplementary Fig. S1). In-depth observations using scanning electronic microscopy revealed that disturbance of DNA methylation induced dramatic morphological alterations at 6 hpf already. A typical alteration seemed to consist in an 'external gastrulation', i.e. instead of invaginating inside the blastocoele, some of the embryonic cells seemed to evaginate outwards (Fig. 4a). After 24 h, most of the control embryos were D-larvae displaying typical traits such as the presence of a velum and of a primary shell with a hinge. In contrast, the 24-hpf 5-aza-C-treated animals consisted in a cluster of mostly the same kind of ciliated cells (Fig. 4b). These animals died within a few hours, indicating an association between an inappropriate early DNA methylation and a deleterious phenotype.

Specific Methylation of Homeobox Gene Orthologues and Corresponding mRNA Expression Levels

The gene-specific methylation profiles and the mRNA expression levels were plotted on the same graphs (Figs. 5 and 6). The selected genes displayed variations in both their specific methylation and mRNA expression level during early development. These two parameters were significantly inversely correlated for seven out of the ten *homeobox* genes investigated ($p<0.05$: *Abd*, *Alx-4*, *Eyegone*, *Hmtx*, *Hoxpost*, *Orthopedia*, *Post2*). Two of them displayed a tendency of being inversely correlated ($0.05<p<0.1$: *Mox2*, *Notochord*), whereas the methylation and expression level of *Prospero* did not seem to be correlated (Fig. 5). In contrast, the situation was less clear for the other early genes investigated outside the *homeobox* family. Indeed, within the orthologues of the PRC group, BMI-1 displayed a '*homeobox*-like' profile, whereas the methylation and expression of EED were positively correlated. Besides, no correlation between specific methylation and mRNA levels could be established neither for other PRC genes (*PHC*, *Sfmbt*, *Suz12*) (Fig. 6a), nor for epigenetic regulators (*JmjCA*, *JmjCB*, *JmjD6*, *KDM2*, *OSA*) (Fig. 6b). This result suggests that, among the examined genes during oyster early development, a direct negative correlation between DNA methylation and transcription would mostly affect *homeobox* genes.

Discussion

This study demonstrates for the first time the functional importance of DNA methylation in the development of a lophotrochozoan species. In addition, it presents the association between specific DNA methylation and gene expression and more specifically its functional role within the oyster development as a possible critical epigenetic regulator of development gene transcriptional activity. Oyster genomic DNA was not constantly methylated during the larval life. Indeed, the methylation profile of the *C. gigas* genome was found partially reminiscent of the situation in the mouse genome, where DNA undergoes a global demethylation promptly after fertilisation (Razin and Shemer 1995) and then becomes re-methylated at specific loci (Hershko et al. 2003). Such changes are thought to enable the establishment of the pluripotent state of cells and the subsequent commitment into

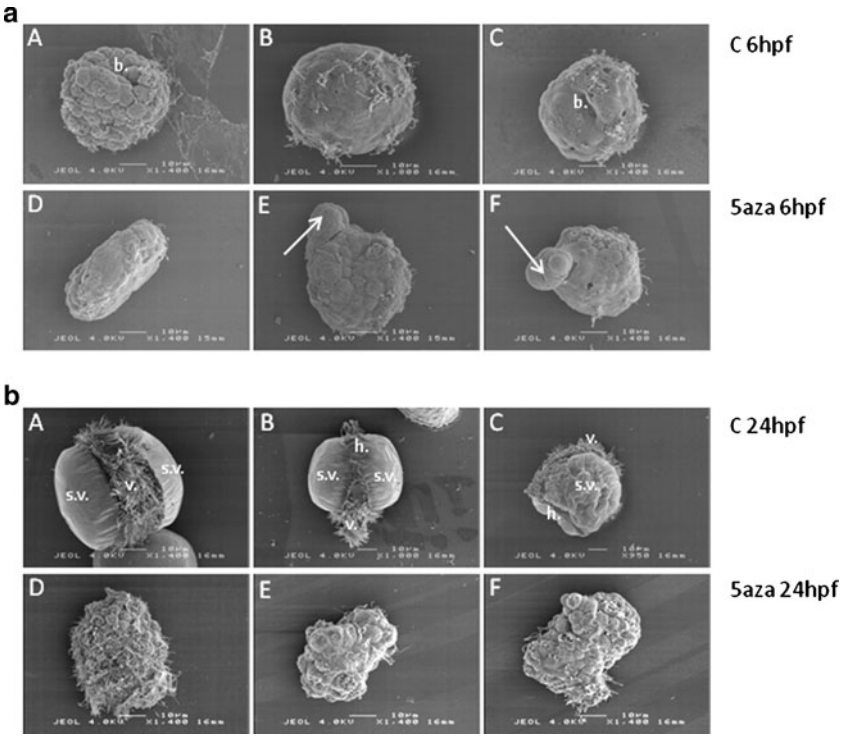

Fig. 4 Influence of 5-aza-C on *C. gigas* embryos at 6 and 24 h post-fertilisation. Scanning electronic microphotographs of oyster embryos in the absence (*A, B, C*) or in the presence (*D, E, F*) of 10^{-5} mol L^{-} 5-aza-cytidine at 6 (**a**) or 24 (**b**)hpf. The scale *bar* (10 μm) and magnification are indicated; *arrows* indicate 'external gastrulation' phenotypes; *b.*, blastopore; *h.*, hinge; *s.v.*, shell valve; *v.*, velum

distinct lineages. However, in contrast to what is observed in mammals, the methylation of the oyster genome is increasing just after fertilisation up to the blastula stage. To our knowledge, this phenomenon has not been reported in other organisms, and its significance remains unclear. However, as in other taxa, a possible role in transposon and/or endogenous gene silencing via direct transcriptional repression or chromatin remodeling could be speculated (review in Smith and Meissner (2013)). From the oyster genome inspection, only the examined DNMTs would be able to methylate DNA. However, unknown enzymes might mediate active demethylation and partly explain the observed kinetics of DNA methylation during embryonic development. Nonetheless, the methylation profile of the oyster genome could be consistent with the expression of the oyster DNMTs orthologues, i.e. DNMT1 and DNMT3b as putative maintenance and de novo methylating enzymes, respectively, when taking into account a possible lag for mRNA translation and enzyme activity within the segmentation period (see (Tomek and Wollenhaupt 2012) for review). Thus, the maximum DNMT1 mRNA level found in oocytes would explain the peak in DNA methylation at the blastula stage, i.e. 3 hpf. Also, the global DNA demethylation is correlated to the following decrease in DNMT1 mRNA levels from the oocytes to the pediveliger larvae and would be related to decreased maintenance methylation. Interestingly, the slight increase in DNA methylation at the trochophore stage could match the peak in DNMT3b expression and might correspond to the de novo methylation wave observed in mammals (Okano et al. 1999). Despite oyster DNMTs being still uncharacterised in terms of cytosine methylation activity, such an interpretation is in line with the conservation of the respective roles of DNMT1 and DNMT3b in the maintenance and de novo DNA methylation in the oyster, as suspected from the high sequence homologies between vertebrates DNMT genes and their oyster orthologues (data not shown). Furthermore, in line with the regulation of cytosine methylation during the larval life, the expression of the MBD orthologues MBD2, MeCP2 and CXXC-1 transcripts also display variations. Similar to their mammalian counterparts, these proteins would mediate downstream outcomes of DNA methylation, such as transcriptional regulation through chromatin remodeling (Brero et al. 2005). Consistent with the finding that DNA is methylated in the oyster genome (Gavery and Roberts 2010), these results strongly suggest that the methylation machinery is conserved in *C. gigas*. Such an observation raises questions about the functional role of DNA methylation, especially during the early development in the pacific oyster.

The importance of cytosine methylation was further assessed by the use of the DNMT inhibitor 5-aza-cytidine. Oyster embryos incubated in the presence of micromolar concentrations of 5-aza-C displayed dramatic alterations and die within 24 to 48 h (Fig. 4). It cannot be excluded that the observed phenotypes partly result from 5-aza-cytidine methylation-independent toxicity. However, the treatment led to a severe decrease in DNA methylation (Fig. 3) and dose-dependent development alterations (see results and supplementary Fig. S1), thereby supporting the functional

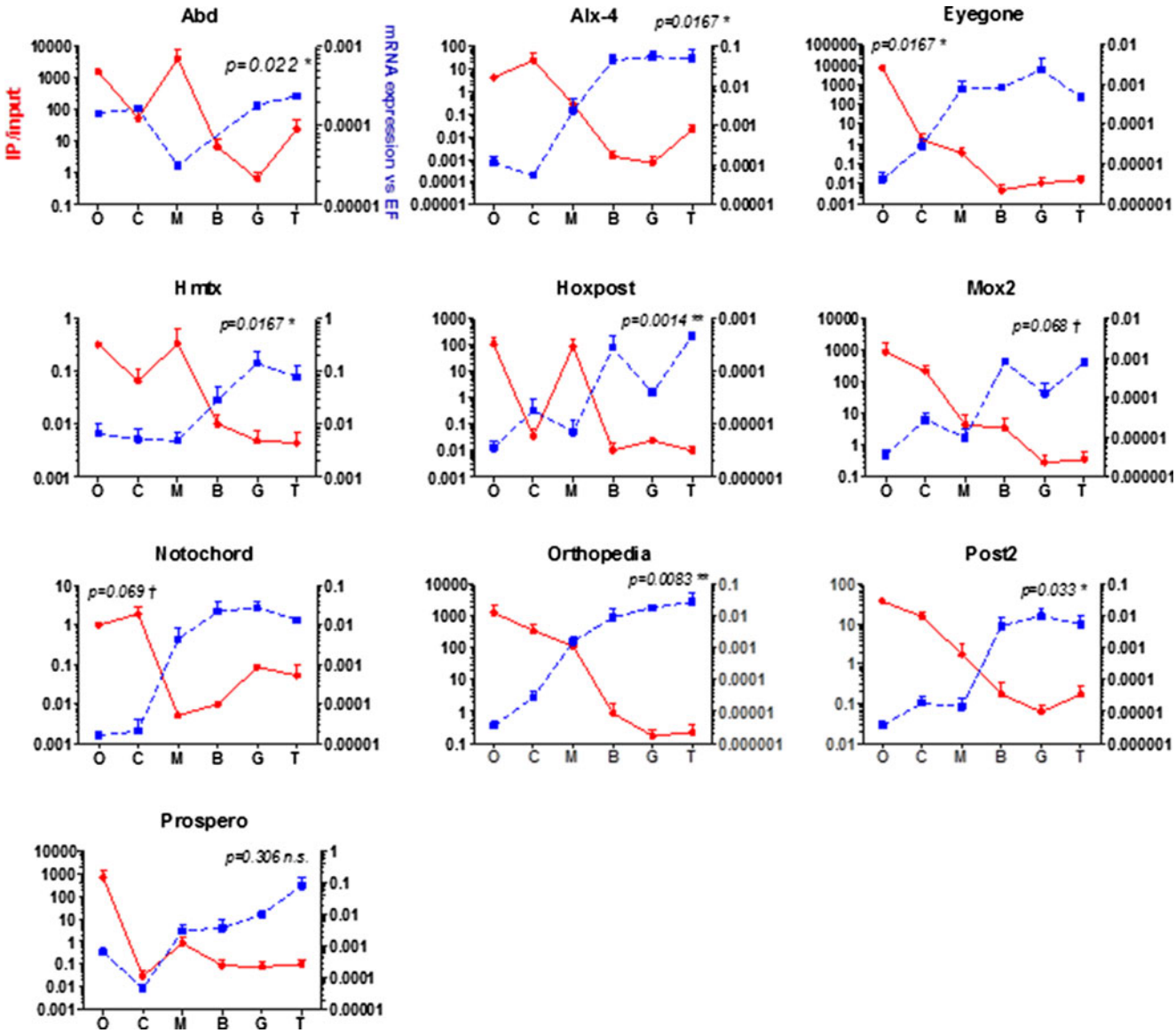

Fig. 5 Influence of gene-specific methylation on *homeobox* genes mRNA expression levels. The methylation (IP/input), *dashed line* (*left Y* axis), and the cognate mRNA expression relative to EF alpha, *solid line* (*right Y* axis) are given in arbitrary units. The development stage is indicated (*X axis*; *O*, oocytes; *C*, two to four cells; *M*, morula; *B*, blastula; *G*, gastrula; *T*, trochophore). The *p* value is given for the negative correlation between gene-specific methylation and mRNA expression. *Plus sign* $0.05<p<0.1$; $*p<0.05$; $**p<0.01$; $***p<0.001$; Pearson or Spearman's correlation test (see "MATERIALS AND METHODS")

significance of DNA methylation in oyster development. Furthermore, treated embryos displayed significant developmental alterations since 6 hpf, which corresponds to the onset of the zygotic genome initiation (review in Tadros and Lipshitz (2009)) but not before. This pattern further suggests that the development defects might arise from altered gene transcription outcomes, likely induced by the dramatic DNA demethylation triggered by 5-aza-C treatment, which was observed in all the embryo pools examined (Fig. 3). Regardless of the demethylation heterogeneity across embryos revealed by 5-mC immunolocalisation, an interesting finding is the observation of an 'external gastrulation' at ca. 6 hpf, a representative trait within the 5-aza-C animal treated group examined, which was not rescued afterwards (Fig. 4a). Such a phenotype differs from an arrested development observed under acute toxicity treatments, therefore further supporting the importance of DNA methylation rather than a direct toxicity of 5-aza-C on the observed developmental outcomes. These phenotypes were hypothesised to be explained by the alteration of cell adhesion properties, as revealed by the electronic microscopy observation of cell surfaces (data not shown). Interestingly, cell adhesion proteins have been demonstrated as downstream targets of *Hox* transcription factors in both *Drosophila* and vertebrates (Van den Akker et al. 2010; Hueber et al. 2007; Jones et al.

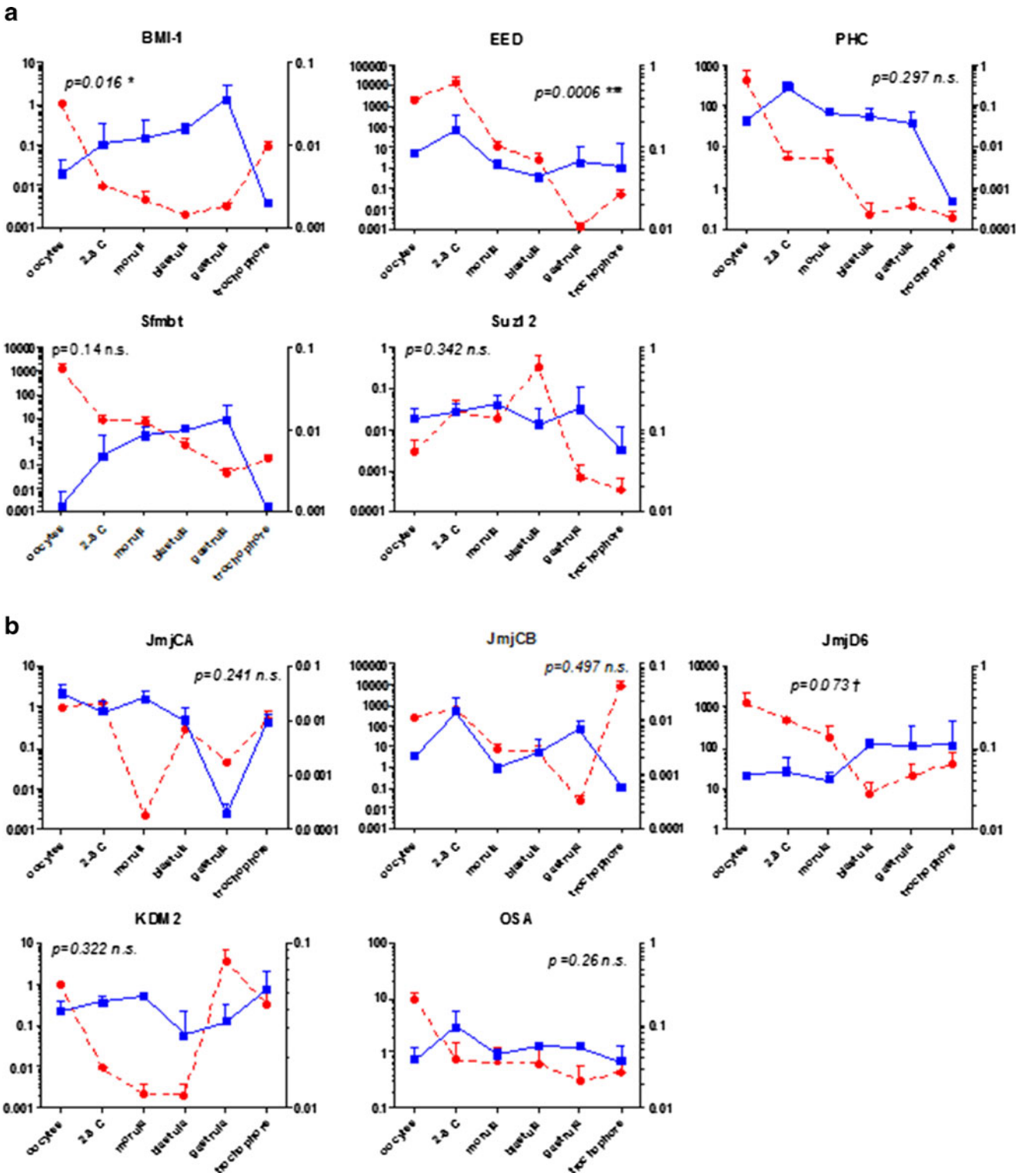

Fig. 6 Influence of gene-specific methylation on mRNA expression levels of PRC genes (**a**) and epigenetic regulators (**b**). The methylation (IP/input) is indicated with a *dashed line* (*left Y* axis), and the cognate mRNA expression relative to EF alpha with a *solid line* (*right Y* axis). The development stage is indicated (*X axis*; *O*, oocytes; *C*, two to four cells; *M*, morula; *B*, blastula; *G*, gastrula; *T*, trochophore). The *p* value is given for the correlation between gene-specific methylation and mRNA expression. *Plus sign* $0.05<p<0.1$; $*p<0.05$; $**p<0.01$; $***p<0.001$; Pearson or Spearman's correlation test (see "MATERIALS AND METHODS")

1992). Later, 5-aza-C-treated embryos at 24 hpf consisted in bunches of subidentical, poorly differentiated ciliated cells instead of D-larvae (Fig. 4b). As a consequence, since normal larvae start to feed after ca. 24 hpf, treated embryos presumably

die because of the failure to establish the proper transcriptomes leading to the establishment of the digestive structures, when all the egg metabolic resources become consummated. Therefore, an altered DNA methylation would be one of the reasons accounting for the death of oysters after the zygotic genome re-initiation. Our observations indicate that this activation could begin at the blastula stage and might constitute a real mid-blastula transition. To our knowledge, such a time point where maternal transcripts are eliminated and zygotic transcripts become required has never been demonstrated in mollusks to date. Nonetheless, the presence of CpG islands in the examined *homeobox* orthologues (Table 1), together with a great CpG content in 'developmental processes' genes (Gavery and Roberts 2010), highlight their putative susceptibility to transient DNA methylation. These considerations contribute to suspect the importance of early genes, including *homeobox* genes, in mediating the consequences of an improper DNA methylation during the early oyster development.

Therefore, the relationship between DNA methylation and *homeobox* orthologue expression was investigated further. However, when this study was initiated, the oyster genome was not characterised, and only a few markers of early development were described. Few of them were demonstrated to be genuine *homeobox* transcription factors susceptible of controlling downstream cascades (Fabioux et al. 2004; Herpin et al. 2005; Kakoi et al. 2008). Putative orthologues of *Brachyury*, *Decapentaplegic* and *Engrailed* (*SkBra*, *SkDpp* or *SkEn,* respectively) were described in a closely related species, the Japanese oyster *Saccostrea kegaki* (Kin et al. 2009). Nevertheless, despite extremely interesting and well-documented, they were not retained in our study. The reason for that was the lack of a unique *C. gigas* EST displaying a significant homology. Alternatively, when a unique sequence could be related to one of those genes with no ambiguity, none of the primer pairs designed within the available sequence exhibited suitable qPCR efficiency in the conditions we used. Consequently, we searched candidates for early genes in the *C. gigas* EST library that exhibited high homology with the cognate orthologue gene (see "MATERIALS AND METHODS") and investigated their expression level in *C. gigas* embryos. The oyster *homeobox* orthologues (*Abdominal* (*Abd*), *Aristaless-4* (*Alx-4*), *Eyegone*, *Homothorax* (*Hmtx*), *Hoxpost*, *Notochord*/*HoxB1*, *Orthopedia*, *Post2 and Prospero*) were chosen according to their homology with *Hox* genes in other taxa (see "MATERIALS AND METHODS"). Polycomb repressive complex (*BMI-1*, *EED*, *Polyhomeotic* (*PHC*), *Sfmbt*, *Suz12*) (see below) and some putative epigenetic regulators (*Jumonji*(*Jmj*)*CA*, *JmjCB*, *JmjD6*, *KDM2*, *OSA*) orthologues were also chosen and investigated for their susceptibility to regulation by DNA methylation. For this purpose, their specific representation within 5-mC-IPed DNA, reflecting their gene-specific methylation, was compared with input genomic DNA. In parallel, the gene-specific methylation was compared with the cognate mRNA expression in the oyster embryonic stages. Interestingly, transcript abundance and DNA methylation were negatively correlated for most if not all (nine out of ten) of the *homeobox* genes examined. In contrast, the elongation factor alpha transcript level was not found to be influenced by DNA methylation. Consequently, these results suggest that DNA methylation might specifically and directly control the expression of some *homeobox* gene orthologues in a 'CpG-island-like' fashion, where methylation of the proximal promoter and the first exon decreases transcription. To test this hypothesis, the genomic context of the target sequences was carefully considered in terms of position within predicted genes and CpG island content (see Table 1). However, it was not possible to undoubtedly reject or confirm this hypothesis due to remaining inaccuracies in the present assembly of the oyster genome (Zhang et al. 2012) (Table 1). In line with a putative methylation-mediated gene silencing, six out of the ten *homeobox* genes investigated were stimulated within embryos upon 5-aza-C treatment (data not shown). The reason why not all the *homeobox* genes examined were stimulated under these conditions could be related to the loss of colinearity of oyster Hox orthologues (Zhang et al. 2012). The polycomb genes examined were stimulated by 5-aza-C (data not shown), whereas their specific methylation was not strongly correlated to transcripts levels. Because DNA methylation in oysters is thought to occur not only in promoter regions but also within gene bodies (Gavery and Roberts 2010), such a regulation could account on distinct CpG content and/or localisation. However, caution should be taken with this interpretation because miRNA and transcription factors might influence the transcription of PRC genes, as described in mammals (Dong et al. 2011; Seo et al. 2011). The following rationale about epigenetic control of oyster development could emerge from the results presented herein. First, the high DNA methylation during the very first cell divisions would block the zygotic genome transcription. Then, DNA would progressively become demethylated through cell divisions, probably because of a decreased maintenance methylation. Thus, zygotic genome would become re-initiated in a 'mid-blastulean transition'-like fashion. In this context, DNA methylation could influence the transcription of specific genes, including some *homeobox* orthologues, through a direct transcriptional derepression and/or through chromatin relaxation. Besides, other epigenetic processes are suspected to impact early genes expression, such as histone modifications (Agger et al. 2009; Terranova et al. 2006), in line with what is known in vertebrates (Laurent et al. 2010; Liang et al. 2011). Nevertheless, only very little is known about DNA methylation in Lophotrochozoa. The mechanisms of transcriptional regulation by cytosine methylation in the oyster are still largely unknown. Genome-wide analysis of this epigenetic mark, together with the elucidation of cell-specific spatio-temporal expression patterns of the candidate genes, could provide extremely interesting insights into the development of the oyster and more

generally of the lophotrochozoans. Furthermore, such data could shed light on the evolution of epigenetic processes participating in the determination of the highly probabilistic events underlying the very first steps of development. Besides, our findings do not rule out a probable role for DNA methylation in later life, such as the maintenance of housekeeping function, the defense against genomic parasites or the adaptation to a changing environment (Regev et al. 1998; Roberts and Gavery 2012). Indeed, it becomes well supported that transient methylation of genes involved in environmental response would increase 'transcriptional opportunities', i.e. alternative splicing and/or fine-tuning of transcription, hence allowing an important phenotypical plasticity which in turn potentiates evolutionary success in changing environments (Roberts and Gavery 2012). We propose that oyster development genes fall into this category. Therefore, they would be under the control of versatile epigenetic inputs undergoing environmental influence, instead of being constitutively expressed. In other terms, a successful development would be triggered by appropriate environmental cues, rather than being the exclusive result of a determined program that has to cope with the conditions and eventually to fail or succeed depending on them. More in-depth genome characterisation together with gene-specific studies would be required to decipher this point. These issues, which lie beyond the scope of the present study, are at the present time considered in our laboratory with the help of high throughput sequencing methods (MeDIP-seq) together with promoter investigations.

Conclusion

Altogether, our results demonstrate that DNA methylation is crucial in the oyster *C. gigas* development. In addition, they raise the hypothesis that such an influence might occur through the epigenetic control of *homeobox* genes. Epigenetic regulation has been either observed or speculated for the *Hox* genes in ecdysozoans and deuterostomes (Agger et al. 2007; Vasanthi and Mishra 2008). However, to the best of our awareness, this study represents not only the first demonstration of association between DNA methylation and gene expression in lophotrochozoans but also brings insights into its functional consequences on the development of the most important shellfish aquaculture resource worldwide.

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