

Specific Expression of BMP2/4 Ortholog in Biomineralizing Tissues of Corals and Action on Mouse BMP Receptor

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Abstract Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β superfamily, and have been identified by their ability to induce bone formation in vertebrates. The biomineral-forming process, called biomineralization, is a widespread process, present in all kingdoms of living organisms and among which stony corals are one of the major groups of calcifying animals. Here, we report the presence of a BMP2/4 ortholog in eight species of adult corals. The synthesis of such a protein by the calcifying epithelium of corals suggests that coral BMP2/4 plays a role in skeletogenesis, making BMP the

first common protein involved in biomineralization among Eumetazoans. In addition we show that recombinant coral BMP2/4 is able to inhibit human BMP2-induced osteoblastic differentiation in mesenchymal C2C12 cells. We suggest that this inhibition results from a competition between coral BMP2/4 and human BMP2, indicating conservation of binding affinity of BMP and its receptor during evolution from corals to vertebrates. Further studies are needed to understand interactions between coral BMP2/4 and its receptors, and, thus, the action of BMP2/4 in adult corals.

Nucleotide sequence of the coral BMP2/4 genes cloned in this study is available in the GenBank under the accession number EU785981 (*Stylophora pistillata*) and EU785982 (*Acropora sp.*).

Keywords Cnidaria · Biomineralization · Bone morphogenetic protein · Molecular evolution

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Introduction

Biomineralization is a widespread process present in all forms of life. Mineralized skeletons appeared for the first time almost “simultaneously” in many groups during the Cambrian Period (Knoll 2003, Lowenstam and Weiner 1989), suggesting a common event at their origin (“Ancient heritage hypothesis”, Marin et al. 2008). Cnidarians are widely accepted as the sister group to all other Metazoans and are thought to be the first Eumetazoa (Martindale et al. 2002, Putnam et al. 2007). We, therefore, focused our work on corals (Cnidarians: Anthozoans) which are responsible, together with calcareous algae (coccolithophores) and foraminifera, for 99.9% of the calcareous deposits on the surface of the globe (Barnes and Chalker 1990).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor- β (TGF- β) superfamily of proteins, which includes TGF- β s, activins, and inhibins (Wozney et al. 1988) and acts as differentiation factors

(Gazzerro and Canalis 2006). In vertebrates, BMPs, originally discovered on the basis of their presence in osteoinductive extracts of bone matrix (Urist 1965), are known to induce the formation of new cartilage and bone when implanted ectopically (for review, see Canalis et al. 2003), demonstrating that members of this class of molecules were necessary and sufficient for osteoinduction (Wang et al. 1990). They have been identified in a wide range of vertebrates (see Wozney 2002 for review) and invertebrate species including worms (Suzuki et al. 1999), flies (Padgett et al. 1987, Wharton et al. 1991), sea urchins (Hwang et al. 1999), mollusks (Nederbragt et al. 2002) and cnidarians (Lelong et al. 2001, Hayward et al. 2002, Hwang et al. 2003). In this phylum, the *dpp/BMP2/4* ortholog is differentially expressed in the larval stage and it is assumed to play a role in tissue differentiation and axis determination (Finnerty et al. 2004, Hayward et al. 2002). However, the potential role of BMP in coral biomineralization was not investigated nor its presence in adult corals. We, therefore, studied the relationship between BMP and biomineralization in adult corals.

In the present study, we determined (1) whether *bmp2/4* was expressed in adult corals from different clades, (2) where *bmp2/4* was expressed in the model adult coral *Stylophora pistillata*, (3) the effect of coral *bmp2/4* on the osteogenic differentiation of C2C12 mouse mesenchymal cell lines. We also discussed the involvement and properties of BMPs in the biomineralization process from cnidarians to vertebrates.

Materials and Methods

Biological Material

Corals used in the present study were cultured in controlled conditions for several years in the laboratory of the Centre Scientifique de Monaco. Eight species, belonging to different genera, were used. Phylogenesis of corals has been based largely on studies of skeletal characters of both recent and fossil corals. Analyses of morphological and molecular characters among the 1,314 known species suggest that reef-building coral are phylogenetically divided in two clades: “complex” and “robust” (Kerr 2005, Le Goff-Vitry et al. 2004, Romano and Cairns 2000). The “robust” corals have solid, heavily calcified skeletons, resulting from the solid construction of corallite walls and forming massive or plate-like structures. The “complex” corals have less heavily calcified skeletons, resulting from the porous construction of the corallite walls showing a light, complex architecture. We focused our studies on four species belonging to the “complex” clade (*Turbinaria*

reniformis, *Acropora* sp., *Pavona cactus*, and *Galaxea fascicularis*) and four species belonging to the “robust” clade (*Hydnophora pilosa*, *Lobophyllia* sp., *Psammocora obtusangula*, and *Stylophora pistillata*).

Cell Culture

C2C12 murine mesenchymal progenitor cells were maintained in DMEM (Life Technologies) supplemented with 10% New-born Calf Serum (NCS, HyClone, Logan, UT, USA), penicillin (100U/ml), and streptomycin (100µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂. For differentiation experiments, cells were seeded at a density of 2.0×10^4 cells/cm² and grown for 24h. Subsequently, medium was replaced by DMEM in the presence or in the absence of 300ng/ml of recombinant human BMP-2 (hBMP-2) with or without 3µg/ml of recombinant coral BMP2/4 (cBMP2/4).

HEK293 (Human Embryonic Kidney) cells (Graham et al. 1977) were maintained in DMEM (Life Technologies) supplemented with 5% NCS.

Molecular Cloning and Expression of cBMP2/4 in HEK 293 Cells

PCR products were prepared from oligo-dT RT coral cDNAs and ligated to the pBAD/Thio TOPO® vector (Invitrogen). PCR reaction was carried out with the following primers: BMPAUGfwd: 5'-ACCATGTTGACCGCTCGACTATG-3' (nucleotides 790–812 of sequence AF285166) and BMPSTO-Prev: 5'-ACGGCAACCACAGCCGTCTAC-3' (nucleotides 1906–1926 of sequence AF285166). The recombinant vector BMPORFHIS contains a chimeric sequence encoding the full-length ORF of the *Acropora* sp. *BMP2/4* fused to a sequence coding a polyhistidine region at the C-terminus. Further PCR experiment using BMPAUGfwd and pBADrev (5'-CTGCGTTCTGATTTAATCTGTATC-3') were performed on recombinant plasmid, and resulting PCR product was cloned into pIRES-DSRED Mammalian expression vector (Clontech, Inc). After sequencing, the plasmid clone was introduced into HEK 293 cells with the Lipofectamine transfection reagent (Invitrogen). A positive clone (HEK-BMP), isolated by limited dilution, was used to produce cBMP2/4.

The H→Q substitution variant was obtained with the method described by Adereth et al. (2005). The phosphorylated primers used to introduce the mutation, changing codon CAG to CAT (His302→Gln302), were as follow: 5'-P-GGCTTTTATTGCAAAGGCGAG-3' (forward primer); 5'-P-ATGATAACCAGGAGGCGCCAC-3' (reverse primer).

Purification of Recombinant cBMP2/4

When the HEK-BMP cells were 70–80% confluent, the supplemented DMEM was replaced with serum-free DMEM; medium was harvested 7 days after. One liter of supernatant of HEK-BMP cell culture was concentrated on a Centricon Plus Centrifugal Filter Unit (30,000MW cut-off). Then, the supernatant was loaded onto a Ni-NTA agarose column, in order to perform affinity purification using the 6× His-tag of the recombinant protein. The Ni-NTA agarose column was washed with buffer 1 (20mM imidazole, 300mM NaCl, 50mM NaH₂P0₄ and 0,05% Tween 20, pH 8.0), and the 6× His-tagged protein was eluted with the buffer 2 (200mM imidazole, 300mM NaCl, 50mM NaH₂P0₄ and 0.05% Tween 20, pH 8.0). The removal of imidazole was accomplished by concentration of eluted protein, then diluting the retentate with PBS using a Centricon Plus Centrifugal Filter Unit (30,000MW cut-off). The process was repeated four times, at the end of which, the retentate was collected and protein concentration was measured using a BCA kit assay (Pierce).

Western Blot Analysis

Proteins extracted from whole coral tissues (15μg of proteins) were resolved by SDS-PAGE (gradient 4 to 20% acrylamide for the resolving gel, 4% acrylamide for the stacking gel). Proteins were then electrophoretically transferred onto PVDF membranes. After transfer, membranes were saturated with 5% skimmed milk in TBS containing 0.1% Tween 20 and labeled for 1h with primary antibody. Membranes were then rinsed and incubated for 1h with secondary antibody (horseradish peroxidase-linked anti-rabbit IgG) and then detection was performed with ECL kit (Pharmacia). Controls were made with the pre-immune serum as primary antibody.

Fluorescence Immunocytochemistry on Tissue Sections

Stylophora pistillata microcolonies were fixed in 3% paraformaldehyde in S22 buffer (450mM NaCl, 10mM KCl, 58mM MgCl₂, 10mM CaCl₂, 100mM Hepes, pH 7.8) at 4°C overnight and then decalcified using 0.5 M EDTA in Ca-free S22 at 4°C. They were then dehydrated in an ethanol series and embedded in Paraplast. Cross-sections (7μm thick) were cut and mounted on silane-coated glass slides. Then sections were incubated for 1h in saturating medium (5% BSA, 0.2% teleostean gelatin, 0.2% Triton X100 in 0.05M PBS pH 7.4) at RT. The samples were then incubated with the anti-SOM (Soluble Organic Matrix) antibodies (Puverel et al. 2005; 1:200 dilution) or cBMP2/4 antibodies (1:250 dilution) as primary antibodies. After rinsing in PBS pH 7.4, samples were incubated with

biotinylated anti-rabbit antibodies (Amersham 1:250 dilution, 1h at RT) as secondary antibodies. Samples were finally washed and labeled for 20min with streptavidin-Alexa Fluor 568 (Molecular probes, 1:50 dilution) and DAPI (2μg/ml, Sigma). Controls were routinely performed without primary antibodies and with rabbit pre-immune serum as primary antibody. Samples were embedded in Pro-Long antifade medium (Molecular probes) and observed with a confocal laser scanning microscope (Leica, TCS4D).

Quantification of Osteoblast mRNA Levels by RT-qPCR

Upon hBMP2 (with or without cBMP2/4) treatment, C2C12 mesenchymal progenitor cells differentiate into cuboidal-shaped osteoblastic cells, whereas they form myotubes in response to low serum conditions without hBMP2 (Katagiri et al. 1994). After treatment, total RNA was isolated from cultured cells using Trizol (InVitrogen). Two micrograms of total RNA were DNase-treated with DNA-free™ (Ambion) and reverse transcribed using the Superscript III RT kit (InVitrogen) with random hexamer primers. Subsequently, cDNA was amplified using SYBR Green PCR Mastermix (Applied Biosystems, Warrington, UK) under the following conditions: initial denaturation for 10min at 95°C followed by 40 cycles consisting of 15s at 95°C and 1min at 60°C. All RT-qPCR reactions were performed in triplicate, and the amplification signal from the target gene was normalized to acidic ribosomal phosphoprotein P0 (36B4) signal in the same reaction (Moya et al. 2008, Mar Biotech). Data are presented as the fold induction in treated cells versus control cells. The sequences of the primer sets for the target genes are shown in Table 1. All experimental data presented were obtained from three independent experiments, each in triplicate, and results are expressed as the mean ± SD of representative experiment. Comparisons between treatments were performed using Student's *t*-test. Values of *p* < 0.05 were considered statistically significant.

Results and Discussion

Expression of BMP2/4 in Adult Coral

Until now, coral BMP2/4 expression had only been shown in coral larvae where it is involved in development (Hayward et al. 2002). As corals are biomineralizing organisms and BMPs are molecules involved in skeletogenesis in vertebrates, we first asked whether BMP2/4 was also present in adult corals and whether it was associated with biomineralization in these invertebrates. We thus performed RT-PCR experiments using mRNA isolated from eight species belonging to the two coral clades,

Table 1 Sequences of the primers used for polymerase chain reaction and sizes of the products

Gene	Forward primer	Reverse primer	Product size, Pb	Reference
36B4	5'-TCCAGGCTTTGGGCATCA-3'	5'-CTTTATCAGCTGCACATCACTCAGA-3'	74	(Beranger et al. 2008)
ALP	5'-TCAGGGCAATGAGGTCACATC-3'	5'-CACAAATGCCACGGACTTC-3'	67	(Rawadi et al. 2003)
OCN	5'-TTTAGGGCGCATTCCATC-3'	5'-TGTCTTGTGGATTAAGGACTTG-3'	101	(Rawadi et al. 2003)
Cbfa1	5'-TTTAGGGCGCATTCCATC-3'	5'-TGTCTTGTGGATTAAGGACTTG-3'	102	(Tan et al. 2007)
Hey1	5'-ACTTGAGTTCGGCGCTGTGTTCC-3'	5'-GCGCTTCTCGATGATGCCTCTCC-3'	89	This study
Hes1	5'-ACAGAAAGTCATCAAAGCCTATCA-3'	5'-TTCTTAAGTGCATCCAAAATCAGT-3'	100	This study

36B4 60S acidic ribosomal protein PO; ALP alkaline phosphatase; OCN osteocalcin; Cbfa1 core-binding factor α 1; Hey1 hairy/enhancer of split related with YRPW motif 1; Hes1 hairy enhancer of split 1

“robust” and “complex” (see “Materials and Methods”). The expected 185-bp amplified DNA was obtained in all species tested (Fig. 1), indicating undoubtedly that BMP2/4 mRNA is expressed in adult corals whatever the clade tested.

In order to localize BMP2/4 proteins in tissues, we raised a polyclonal antibody. We, thus, cloned the ORF sequence of two coral species: a “complex” coral *Acropora sp.* and a “robust” coral *Stylophora pistillata*. Both clones were sequenced showing weak differences in ORF nucleotide sequence (GenBank accession number EU785982-*Acropora* and EU785981-*Stylophora*). The ORF cDNA of *Stylophora pistillata* was inserted in a mammalian expression vector and transfected in HEK293 cells (HEK-BMP). Production of cBMP2/4 was analyzed by using an antiserum directed against a 6 \times His-tag genetically fused to cBMP2/4 gene (Fig. 2). As with all TGF- β family member, BMP2/4 protein is synthesized as a pre-protein. Then, processing of BMP2/4 in its active form involves glycosylation, homodimerisation, and cleavage by pro-protein convertases, such as furin (Cui et al. 1998). We have no evidence that convertases exist in coral but cBMP2/4 protein possesses the amino acid motif RRRKR (aa 257–261 in *Stylophora*). As expected by comparison with other BMPs (Wang et al. 1990), anti-His tag reveals by Western blotting an intracellular coral recombinant pro-protein near 60kDa (Fig. 2 lane 1) and a secreted form of 18kDa (Fig. 2 lane 2) suggesting that it is processed correctly in HEK cells. The secreted purified recombinant cBMP2/4 was used to produce an anti-cBMP2/4 antiserum (Fig. 2 lane 5). This antibody was also tested on the tissue extract of eight species of corals (Fig. 2 lane 6 to 13). The cBMP2/4 antibody recognized the 60-kDa intracellular precursor protein in all tested species since the mature form is processed outside the cells (Israel et al. 1992). Furthermore, tissues sections from *Stylophora pistillata*, which is often used as a model for biomineralization studies (Allemand et al. 2004) and has a well-established structural organization (Tambutte et al. 2007), were labeled with the anti-cBMP2/4 antibody and stained with DAPI (Fig. 3). Anti-SOM antibody which specifically labels the calico-

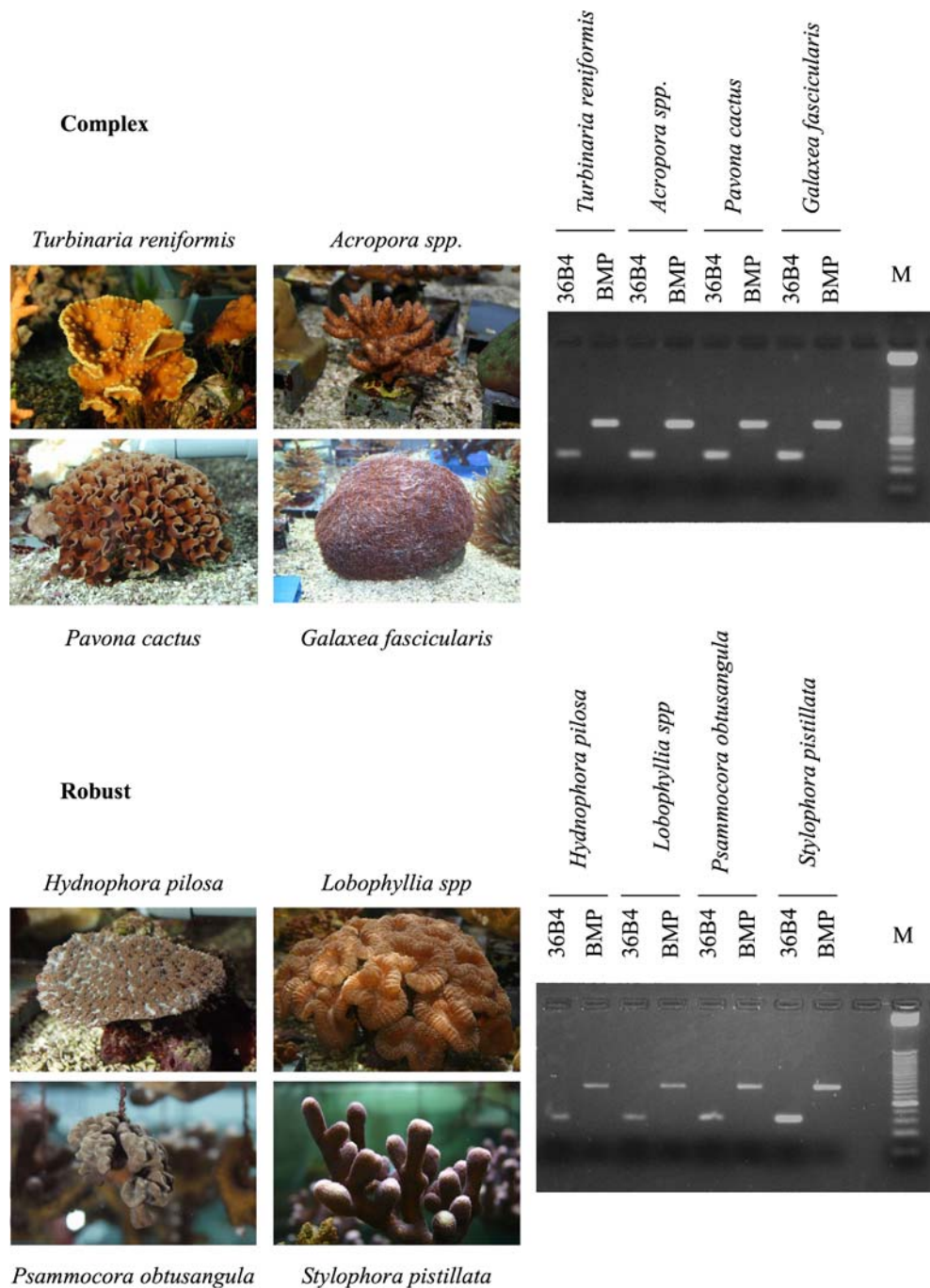
blastic ectoderm (Puverel et al. 2005) was used as positive control (Fig. 3b). As can be seen in Fig. 3b, the cBMP2/4 antibody specifically labels the calcicoblastic ectoderm, with no signal within other cell layers (oral ectoderm and endoderm or aboral endoderm) nor within the extracellular matrix (mesoglea). Observation at higher magnification showed that labeling is both cytoplasmic and associated with cell membranes indicating that these cells secrete BMP2/4 towards the skeleton (Fig. 3b, inset). The pre-immune serum gave no signal (results not shown).

Thus, our results show that cBMP2/4 is present in adult coral. Its expression is not restricted to a single coral species, but exists in all eight reef-building corals tested in the present study. In planulae of the sea anemone *Nematostella*, Finnerty et al. (2004) have shown that cBMP2/4 is expressed in both the gastrodermis (endoderm) and the ectodermal area of the apical organ at the aboral end involved in the settlement of the animal. Interestingly, after settlement, this ectodermal area will differentiate into calcicoblastic ectoderm, which is involved in skeletogenesis (Allemand et al. 2004, for review). Our results demonstrate that in adults, cBMP2/4 is specifically synthesized by this calcicoblastic epithelium. Therefore, cBMP2/4 might play a role in biomineralization as a signaling molecule on coral stem cells in order to ensure their differentiation into calcifying cells.

Bone-forming Activity of Coral BMP2/4

In mammals, it has been shown that BMP2/4 acts on cells by binding to a receptor triggering an intracellular signaling pathway leading to the differentiation of osteoblast cells (Gazzerro and Canalis 2006). To further investigate if cBMP2/4 is recognized by the mouse BMP receptor, the bone-forming activity of recombinant cBMP2/4 was assayed. To study the differentiation process at the transcriptional level, we analyzed mRNA expression of a number of molecular markers of osteoblast differentiation, using the murine mesenchymal progenitor cell line C2C12 as a model system. These cells have been described

Fig. 1 Amplification of cBMP2/4 gene fragments from eight coral species. Four species from the “complex” clade and four species from the “robust” clade were chosen on the basis of their diversified morphological characters. After extraction and DNase-treatment, RNA was reverse transcribed and submitted to PCR with 60S acidic ribosomal protein P0 primers (36B4), as positive control gene expression (Moya et al. 2008), or cBMP2/4 primers (BMP). Amplifications were subjected to a 3% agarose gel electrophoresis giving a 75-bp band for 36B4 and a 185-bp band for BMP. *M* 25-bp DNA ladder from InVitrogen

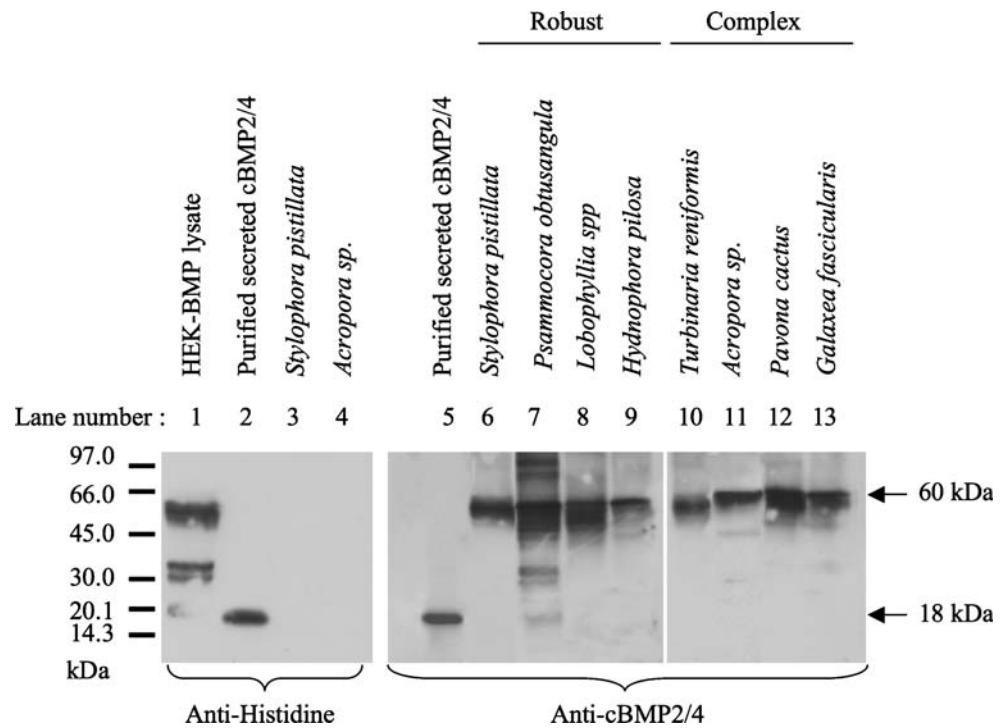


previously for their ability to undergo BMP-2-induced differentiation into cells of the osteoblastic lineage (Katagiri et al. 1994). When cultured in medium-supporting low mitogenic activity, C2C12 cells form multinucleated myotubes after reaching confluence. However, this process can be inhibited completely by culturing the cells in the presence of human BMP2 (hBMP2), resulting in the expression of osteoblast markers such as alkaline phosphatase (ALP) and osteocalcin (OCN; Katagiri et al. 1994). This process is driven by a complex network of specific transcription factors such as *Runx2/Cbfa1* (Ducy et al.

1997) and a member of the Notch signaling pathway, *Hey1* (Iso et al. 2001). C2C12 cells (Fig. 4) were incubated with cBMP2/4, in combination or not with hBMP2 (H+C or C respectively), for 3days and compared with non-stimulated time matched (control) or hBMP2 (H as positive control).

Firstly, mRNA expression of the osteoblast phenotypic marker genes ALP and OCN was examined by real-time PCR. Although it is well established that hBMP2 induces the expression of ALP and OCN (Vaes et al. 2002), we observed that cBMP2/4 does not induce the expression of both markers in C2C12 cells (Fig. 4 panels a and b). On the

Fig. 2 Immunoblot analysis of cBMP2/4. After electrophoresis on SDS-PAGE and transfer on PVDF membrane, proteins of lysate of HEK-BMP, purified cBMP2/4 or lysate of eight species of corals were probed with either anti-Histidine tag or anti-cBMP/4. Pre-protein of about 60 kDa or secreted mature protein of about 18 kDa, recognized by antibodies, are indicated by *arrows*



contrary, surprisingly, cBMP2/4 inhibits the hBMP2-induced expression of both genes (panels a and b). The results may explain the lack of osteoinductive properties of coral skeleton when it is used in bioimplants (Begley et al. 1995; Shahgaldi 1998). Indeed, coral is one of the few biomaterials which has been extensively studied as a bone graft substitute, with animal and human research investigations being conducted for almost 30years (Demers et al. 2002).

Secondly, we tested the expression of osteoblastic specific transcription factor genes *Runx2/Cbfa1* and *Hey1*, known to be differentially regulated by BMP2 (de Jong et al. 2004a; de Jong et al. 2004b). As can be seen in Fig. 4 panel c, *Hey1* is up-regulated by hBMP2. As previously shown for ALP and OCN genes, cBMP2/4 does not induce *Hey1* expression and inhibits hBMP2 induction. In the same manner, as shown in Fig. 4 panel d, cBMP2/4 is unable to induce *cbfa1* expression in contrast to hBMP2.

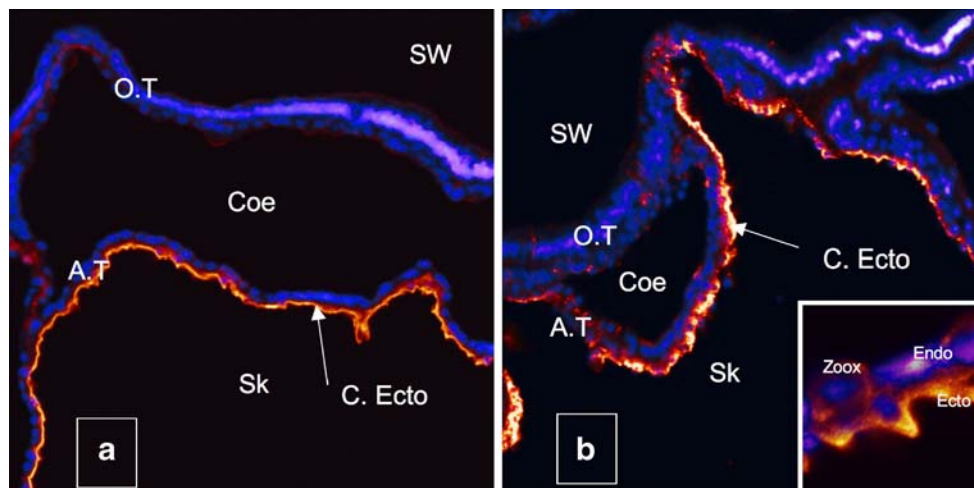
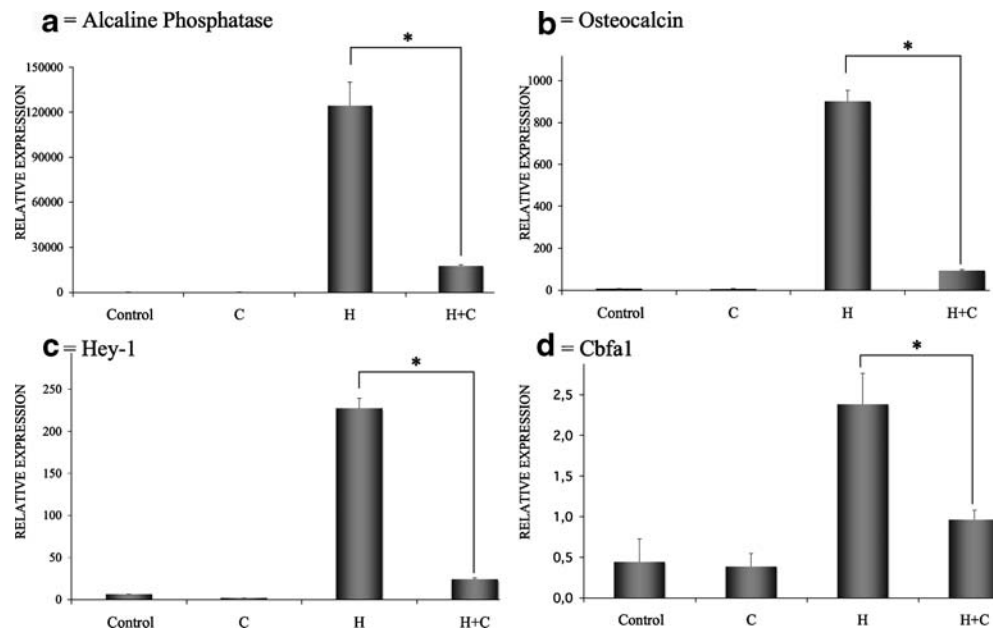


Fig. 3 Immunolabeling of tissues from *S. pistillata* embedded in Paraplast. **a** Positive control immunolabeling with anti-SOM antibody (orange) merged with DAPI staining of nuclei (blue to white) showing specific labeling of calicoblastic cells in the coenosarc; **b** immunolabeling with anti-BMP2/4 antibody showing also specific labeling of calicoblastic

cells in the coenosarc. *Inset* shows a magnification of aboral layer showing that labeling in calicoblastic cells is cytoplasmic. *SW* seawater side, *SK* skeleton side, *Coe* coelenteron, *O.T* oral tissue, *A.T* aboral tissue, *C. Ecto* calicoblastic ectoderm, *Zoox* zooxanthellae, *Endo* endoderm, *Ecto* ectoderm

Fig. 4 Differential regulation of expression of different osteoblast marker genes by hBMP2 and cBMP2/4 on C2C12 cells. Cells were seeded and grown for 24 h upon which the medium was replaced by control DMEM (*Control*), DMEM+cBMP2/4 (*C*), DMEM+hBMP2 (*H*), or both factors (*H+C*). RNA was isolated from cells 3 days after stimulation. Expression levels of alkaline phosphatase (panel **a**), osteocalcin (panel **b**), *hey1* (panel **c**), or *cbfa1* (panel **d**) were measured by real-time quantitative RT-PCR. Mean expression levels were corrected for 36B4 levels. Data represent the mean±SD of representative experiment



Furthermore, the cBMP2/4 inhibits the hBMP2 effect. All these results indicate that cBMP2/4 acts as an antagonist of hBMP2 osteogenic induction in mammals.

These results actually raise the question of the mechanism of action of cBMP2/4 on C2C12 cells. The first hypothesis (Fig. 5a) is that coral BMP acts *via* the TGF- β receptor since it has been previously shown that hBMP2-induced osteoblast differentiation can be blocked by TGF- β (de Jong et al. 2004a; Lee et al. 1999). In our experiments, if cBMP2/4 interacts with the TGF- β receptor, then cBMP2/4 would counteract the hBMP2 effect. Effectively, as shown in Fig. 4, we observed the inhibition of ALP, OCN and *hey1* up-regulation. However, on the contrary to TGF- β (Lee et al. 1999), coral BMP2/4 is unable to induce *cbfa1* expression. Our results, thus, invalidate the first hypothesis.

The second hypothesis (Fig. 5b) is that coral BMP acts as a receptor antagonist on BMP receptors. It has been shown that hBMP2 interacts with two receptors on C2C12 cells, BMPR-IA and BMPR-II which form a heteromeric complex. Upon BMP binding, the BMPR-II transphosphorylates the BMPR-IA, which transmits intracellular signals (Nohe et al. 2004). By experiments on mutation variants, two receptor-binding motifs (epitopes) have been identified in BMP2 (Kirsch et al. 2000), one interacting with BMPR-IA, the other with BMPR-II. When comparison is done between human and coral BMP, there is no difference in epitopes involved in the binding with BMPR-IA (Fig. 5c). These observations lead us to suggest that cBMP2/4 could bind to BMPR-IA and compete with the binding of hBMP2 on the BMPR-IA (Fig. 5b). However, there are differences in epitopes involved in the binding with BMPR-II (Kirsch et al. 2000). This discrepancy (histidine residue instead of

glutamine) is present in the three cloned coral BMP2/4 genes. The antagonist effect could be due to a decrease or absence of cBMP2/4 binding on BMPR-II. In this case, BMPR-II would not transphosphorylate the BMPR-I and thus intracellular signals could not be transmitted. However, when we used a cBMP2/4 variant, obtained by H \rightarrow Q substitution, cBMP2/4 inhibitory activity still persists (data not shown). This comparative approach suggests that other amino-acids, currently not identified, are involved in the signal transduction of BMP in mammal's cells. Although we did not find evidence that cBMP2/4 induces any osteogenic signal after binding to mouse BMP receptor, we cannot determine cBMP2/4 action on adult coral cells since interaction studies between cBMP2/4 and its receptors are needed.

It is noteworthy that the cnidarian BMP2/4 is active on *Drosophila* (Hayward et al. 2002) or zebrafish (Rentzsch et al. 2006) receptors, showing the importance of studies on the evolution of structure/function of molecules. Although the TGF- β superfamily exists in all animals, from sponges to vertebrates, it seems that BMP2/4 homologues (Hwang et al. 2003) as well as BMP receptors (Suga et al. 1999) arose after the Porifera (sponge)/(Cnidaria+ bilateral Metazoa) split because BMP2/4 homologues are not found in sponges. In Cnidarians, the molecular characterization of a BMP type I receptor and two receptor-activated SMADs, involved in BMP signaling, was reported (Samuel et al. 2001). In silico data mining indicates that BMPR-II is expressed in the sea anemone *Nematostella vectensis* (partial sequence clone XM_001633392). Up to now, it seems that no reports have been published on the location of BMP receptors in corals. Further studies are needed to understand interactions between cBMP2/4 and its receptors, and, thus, the action of BMP2/4 in adult corals.

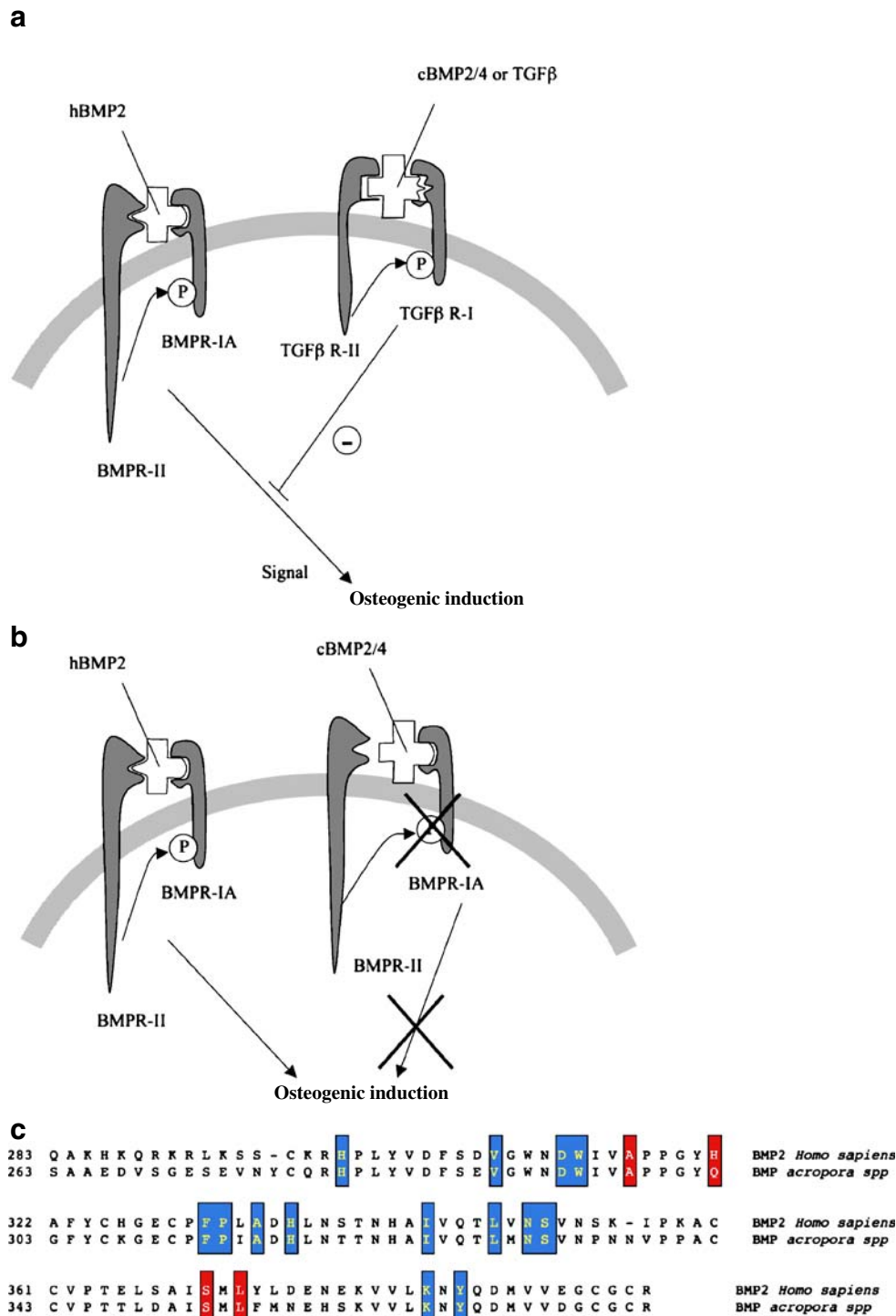


Fig. 5 Possible mechanisms of coral BMP2/4 action on C2C12 cells. **a** Hypothesis concerning the binding on TGF-β receptors. Coral BMP2/4, acting on TGF-β receptors should induce a negative control on hBMP2 pathway. **b** The competition binding hypothesis. Coral BMP2/4 should compete on BMP2 type I receptor binding but should

not trigger signal inside the cells because it should not bind the type II receptor. **c** Comparison between human and coral BMP in the region involved in binding with BMPR-I (blue boxes) and BMPR-II (red boxes). Only one discrepancy is revealed in the region involved in the BMPR-II binding region (H→Q)

Our results clearly support the “ancient heritage” hypothesis of the origin of biomineralization. This hypothesis suggests that some components involved in biomineralization may be ancient and recruited early in the evolution of

bilaterians (Marin et al. 2008). Thus, the BMP-signaling system, putatively present in the common ancestor of Eumetazoans, may have been selected as a key mechanism in biomineralization and, thus, conserved through evolution.

This hypothesis is reinforced by the fact that a role of BMPs in biomineralization of mollusks has also been suggested (Lelong et al. 2000; Nederbragt et al. 2002; Westbroek and Marin 1998).

In conclusion, our study is the first that shows the presence of BMP in adult corals. Furthermore, the synthesis of such a protein by the calcicoblastic cells, responsible for carbonate calcium deposition (Allemand et al. 2004), suggests a role of coral BMP2/4 in coral biomineralization. In addition to its role in determination of body axis in larval development (Finnerty et al. 2004; Hayward et al. 2002), we suggest that the remarkable conservation of the BMP/ligand binding specificity is due to its conserved role in biomineralization from the first eumetazoan to vertebrates, making BMP the first common protein involved in biomineralization among Eumetazoans. The study of coral BMP2/4, a molecule which seems to have appeared in the common ancestor of Cnidarians and bilaterians, and its potential implications in coral skeleton formation, might open new pathways not only in bone graft substitute surgery but also in evolutionary studies of biomineralization and hormone/receptor partnership.

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