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Expression of cBMP4 protein and anti-cBMP4 monoclonal antibody production

Xuezhi Li
San Jose State University

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EXPRESSION OF CBMP4 PROTEIN
AND ANTI-CBMP4 MONOCLONAL ANTIBODY PRODUCTION

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Xuezhi Li

May 2008

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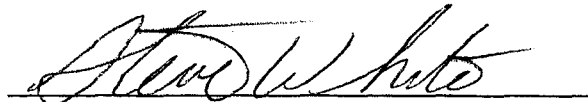
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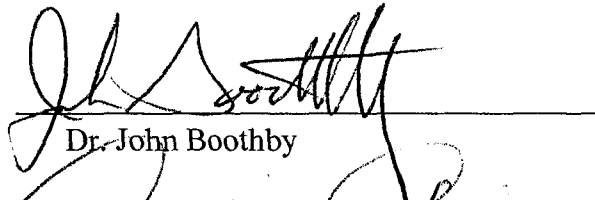
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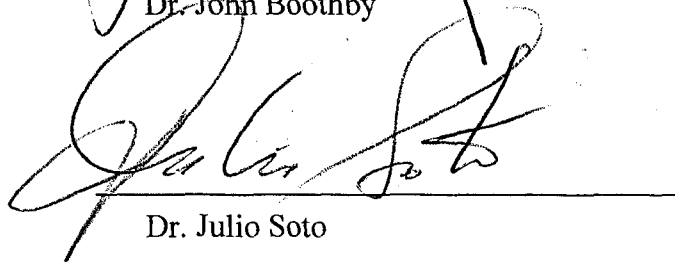
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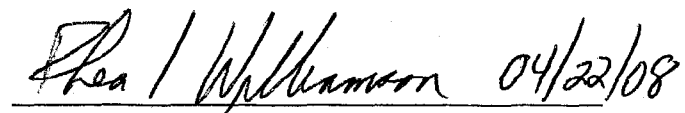
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ABSTRACT

EXPRESSION OF CBMP4 PROTEIN AND ANTI-CBMP4 MONOCLONAL ANTIBODY PRODUCTION

by Xuezhi Li

Malformations during cardiac valve development are some of the most common congenital heart defects. Recent studies on BMP4 in heart development reveal that this secreted protein plays key roles in cardiac cushion formation, differentiation and cushion cells apoptosis. While a few papers have investigated BMP4 transcription patterns at various times during heart development, a clear mapping of cardiac BMP4 expression domains has yet to be accomplished. Studies at the protein levels are particularly hampered in the chick by the lack of suitable specific anti-cBMP4 antibodies. To address this need, chick recombinant BMP4 was cloned, expressed and purified in both COS-7 cells and in *E. coli* for anti-cBMP4 monoclonal antibody (mAb) production. In addition, a BMP4 synthetic peptide was used to produce anti-cBMP4 mAb that could be used for future immunohistochemistry studies to map the spatio-temporal pattern of BMP4 protein expression during heart development.

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Introduction

Congenital Heart Disease

Congenital heart malformation is the most common human birth defect and the leading cause of death in the first year of life (Hoffman, 1995). It is estimated that the US population of adults with congenital heart disease was about 8,000,000 in 2000 (Warnes, Liberthson, & Danielson, 2001). Most congenital heart malformation is due to the defects of septation or valvogenesis events during embryonic heart development. For example, one of the most frequently diagnosed disorders is atrioventricular canal defect (AVCD), which accounts for 7.3% of all congenital heart abnormalities (Pierpont, Markwald, & Lin, 2000). AVCD covers a spectrum of abnormalities. The partial form of AVCD is caused by a deficiency in atrial septum primum (ASP) formation, whereas the complete form is due to the absence of AV septum, resulting in the formation of a single common atrioventricular canal (Digilio, Marino, Giannotti, Di Donato, & Dallapiccola, 2000). The process of embryonic cardiogenesis has been extensively studied at both the anatomical and molecular levels using animal models, enhancing our understanding of the mechanisms underlying cardiac morphogenesis and the pathogenesis of congenital heart defects.

Embryonic Heart Development

The heart is the first organ to form and function during vertebrate embryogenesis. Heart development is a complex process that involves a series of cellular migrations, fusions, and specific differentiations. Due to the evolutionary conservation of many of these processes, major insights of embryonic cardiogenesis have been gained from the

studies of chick embryos. In the pregastrula chick embryo, cells that contribute to the heart are found within the posterior half of the epiblast (Hatada & Stern, 1994; Rawles, 1943). During early gastrulation stage, cardiac precursor cells migrate to a region of the primitive streak caudal to Hensen's node. Thereafter, they migrate into the mesoderm and spread anterolaterally, resulting in the formation of right and left precardiac mesoderms (Garcia-Martinez & Schoenwolf, 1993; Rosenquist & DeHaan, 1966). At Hamburger and Hamilton (HH) 6, the bilateral regions then condense, which later forms a cardiac crescent that subsequently coalesces at the ventral midline to form a linear heart tube. The heart tube is composed of a thin outer myocardium and an inner endocardium. The myocardium secretes a thick extracellular matrix called the cardiac jelly that separates the myocardium and endocardium (Kirby, 2002). The heart tube expands and bends to the right side of the embryo and later the heart tube twists dramatically and forms a d-loop. As the development proceeds, the heart tube dilates, elongates and twists in a right-handed manner, realigning the prospective chambers so that the forming atrium is to the left and cephalic to the ventricle. At the same time, presumptive cardiac segments begin to appear, which make up the conus cordis or outflow tract (OT), primitive right and left ventricles, atrioventricular canal (AVC), atrium, and the sinus venous. After the completion of the d-loop, some of the endocardial cells of the OT and AV segments of the single heart tube change their phenotype to that of mesenchymal cells. These then migrate into the adjacent cardiac jelly to form endocardial cushion tissue, the primordial of the valves and cardiac septa of the adult heart. This embryonic phenomenon involves what is called epithelial-mesenchymal transformation (EMT). At

this time, ectomesenchyme originating from the cardiac neural crest migrates into the cardiac OT via the arterial pole. Thereafter, the endothelially derived mesenchyme and the neural crest-derived mesenchyme develop the outflow septum, semilunar valves and the aorticopulmonary septum, respectively. The AV endocardial cushion tissue develops to form the leaflets of the AV valves and the AV septum. As development proceeds, the aorticopulmonary septum, outflow septum, interventricular septum (muscular ridge of the interventricular foramen), AV septum, and primary atrial septum come into alignment and fuse with each other, resulting in the completion of the four-chambered heart. At this point the heart resembles its mature form (Larsen, 2001; Nakajima, Yamagishi, Hokari, & Nakamura, 2000).

TGF- β Superfamily and Bone Morphogenetic Proteins

The regulatory factors involved in the process of septation and valvogenesis are heavily investigated, with numerous studies indicating that members of the transforming growth factor- β (TGF- β) of signaling molecules are involved. The TGF- β superfamily is composed of over 30 structurally related members with diverse functions in development. Based on sequence homology and signaling mechanisms, the TGF- β superfamily includes i) the TGF- β family, ii) the activin family, iii) the bone morphogenetic proteins (BMPs), iv) the Vg1 family, and v) other proteins, including glial-derived neurotrophic factor and Müllerian inhibitory factor (Hogan, 1996; Gilbert, 2003). The protein encoded by TGF- β superfamily gene is synthesized as a large pre-protein, consisting of a hydrophobic signal peptide, an N- terminal pre-domain and a C-terminal biologically active peptide (Herpin, Lelong, & Favrel, 2004). After protein processing, only the

carboxy-terminal region contains the active mature peptide with the TGF- β domain. Then the mature peptides are dimerized into homodimers (with themselves) or heterodimers (with other TGF- β peptides) and are secreted from the cell for various functions (Gilbert, 2003).

The BMPs constitute the largest family. They were originally identified from bone matrix using an ectopic bone formation assay (Wozney, 1992). To date, around 20 BMP family members have been identified and characterized. Based on sequence homology, there are six main groups: i) the Dpp subgroup, related to *Drosophila* decapentaplegic (dpp), consists of BMP2 and BMP4; ii) the 60 A subgroup, related to *Drosophila* 60 A protein, consists of BMP5, BMP6, BMP7, BMP8 (Zhao, 2003); iii) BMP3A and BMP3B; iv) BMP9 and BMP10; v) BMP12, BMP13 and BMP14, and vi) BMP11 and growth/ differentiation factor 8 (Chen, 2004).

BMPs are 30-38 kD homodimers that are synthesized as prepeptides of approximately 400-525 amino acids. The mature C terminal domain of BMPs usually contains 100- 140 amino acid residues after protein processing and dimerizes for bioactivity. BMP proteins act through type I and type II receptors that are single-pass transmembrane proteins with serine/threonine kinase domains on the cytosolic side of the plasma membrane. Both type I and type II receptor are required for signaling. Three types of type I receptors have been shown to bind BMP ligands, and they are type IA receptor (BMPRI1A or ALK3), IB receptor (BMPRI1B or ALK6) and type IA activin receptor (ActRI1A or ALK2)(Koenig et al., 1994, Macias-Silva, Hoodless, Tang, Buchwald, & Wrana, 1998; Ten Dijke et al., 1994). Three type II receptors for BMPs

have also been identified and they are type II receptor (BMPR II), type II and IIB activin receptors (ActR II and Act R IIB). Whereas BMPR1A, BMPR IB and BMPR II are specific to BMPs, ActR1A, ActR II and ActR IIB are also signal receptors for activins (Kawabata, Chytil, & Moses, 1995; Yamashita et al., 1995). Signal transduction through serine/threonine kinase receptors has been best characterized in the TGF- β receptor system. It is likely that BMP receptors transduce signals in a similar fashion. Typically, the ligand first binds to and activates a type II receptor homodimer which recruits, phosphorylates, and activates a type I receptor homodimer, forming an active tetrameric receptor complex. The type I receptor directly binds and phosphorylates a group of regulatory proteins belonging to Smad family. In mammals, eight members of the Smad protein family have been identified, and these have been grouped into three different classes based on structures and functions (Balemans & Van Hul, 2002). One class of Smads consists of the receptor-regulated Smads (R-Smads) that act as latent transcription factors. R-Smads transiently associate with the type I receptor and are phosphorylated on their C-terminus by the type I receptor kinase domain. Phosphorylated R-Smads then interact with the co-Smad, Smad4, resulting in translocation of the heteromeric complex to the nucleus where it interacts with cell-specific transcription factors to induce target gene expression (Piek, Moustakas, Kurisaki, Heldin, & Ten Dijke, 1999). Binding of different TGF- β family members to their respective receptors results in the activation of different R-Smads. Smads2 and 3 are specifically activated by activin/ TGF- β signaling, whereas Smads 1, 5 and 8 are activated upon BMP stimulation. The last class of Smads consists of the inhibitory Smads, Smads6 and Smads7, which function as negative

regulators of signaling. Smad6 primarily regulates BMP signaling by competing with Smad4 for binding to activated Smad1, resulting in the formation of an inactive Smad6/Smad1 complex (Hata, Lagna, Massagué, & Hemmati-Brivanlou, 1998). Smad7, which lacks the conserved C-terminal phosphorylation site of R-Smads, is unable to translocate to the nucleus and downregulates BMP, activin, and TGF- β signaling by inhibiting the phosphorylation of R-Smads through competition with R-Smads for type I receptor binding (Hayashi et al., 1997).

Bone Morphogenetic Protein 4 and Functions

One member of BMP family is bone morphogenetic protein 4 (BMP4). It is a 405 residue pre-protein in the chick. It contains a signal peptide region from residue 1 to 19, a pre-peptide region from residue 20 to 291 and a mature chain (cleaved post-translationally) from residue 292 to 405, comprising an active carboxyl-terminal peptide of 114 residues. There are four N-linked glycosylation sites at residue 114, 208, 343 and 362 respectively. Each BMP4 monomer is held together by disulphide bridges between three pairs of cysteines in a conformation called the cysteine knot. BMP4 is a secreted protein and its structure is closely related to BMP2. Thus, BMP4 is also named BMP2B.

BMP4 plays an important role in embryogenesis. Early studies using *Xenopus* embryos indicate that BMP4 is a powerful ventralizing molecule. If the mRNA for BMP4 is injected into *Xenopus* eggs, all the mesoderm in the embryo becomes ventrolateral mesoderm, and no involution occurs at the blastopore lip (Dale, Howes, Price, & Smith, 1992; Jones, Lyons, Lapan, Wright, & Hogan, 1992). Conversely, overexpression of a dominant negative BMP4 receptor results in the formation of two

dorsal axes (Graff Thies, Song, Celeste, & Melton, 1994; Suzuki et al., 1994). When BMP binds to ectodermal cells, it activates the expression of genes such as *msx1*, which induce the expression of epidermal-specific genes, while inhibiting those genes that would produce a neural phenotype (Suzuki, Ueno, & Hemmati-Brivanlou, 1997). The mechanism of ventral ectoderm (epidermis) induction is depended on the formation of a BMP4 gradient. The gradient of BMP4 activity, largely established by organizer-secreted BMP4 antagonists such as chordin, noggin, and follistatin through binding and thus inhibition of BMP4 activity (Dale & Wardle, 1999; Harland & Gerhart, 1997) specifies the different mesodermal territories along the marginal zone of the *Xenopus* gastrula. In particular, high levels of BMP4 activity specify ventral mesoderm fates such as blood, intermediate BMP levels specify more lateral mesoderm derivatives such as kidney, and low levels of BMP activity (in the region closest to the organizer) specify dorsal fates such as muscle (Winnier, Blessing, Labosky, & Hogan, 1995).

A large number of studies in several different species have implicated a key role of BMP4 hematopoietic commitment. Huber et al. (1998) has demonstrated that BMP4 together with either basic fibroblast growth factor or activin A can induce the formation of red blood cells from non-mesodermal structures using *Xenopus* embryos and explant assays. Furthermore, hematopoietic commitment induced by the GATA binding transcription factors GATA1 and GATA2 requires intact BMP signaling (Huber, Zhou, Mead, & Zon, 1998; Maceno et al., 1996). Mice lacking BMP4 died between embryonic day 7.5 to 7.9 (E7.5 to E9.5) exhibiting severe defects in mesoderm formation and the embryos that survive up to E9.5 show defective blood islands (Winnier et al., 1995). In

addition, it is recently demonstrated that BMP4, in combination with cytokines, promotes hematopoietic differentiation of human embryonic stem cells (Chadwick et al., 2003). BMP4 may act as an important positive regulator of both proliferation and survival of human stem cells (HSCs). Bhatia et al. (1999) found that low concentration of BMP4 promoted proliferation and differentiation of CD34⁺CD38⁻Lin⁻ cells while high concentration of BMP4 extended the duration of stem cell activity in Nonobese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice transplanted with purified HSC (CD34⁺CD38⁻Lin⁻) from human hematopoietic tissue following *ex vivo* culture in serum free medium. Thus BMP4 is acting as a survival factor, preserving the stemness of the hematopoietic cells.

BMP4 is also involved in organ development. The lung appears from the ventral foregut as two primary buds, each consisting three layers: the inner epithelium (of endodermal origin), the surrounding mesenchymal stroma, and a thin outer mesothelium. Early in development, the epithelium undergoes a stereotypic pattern of branching morphogenesis to form the conducting airways, a process regulated by epithelial–mesenchymal interactions (Cardoso, 2001). *In vitro* studies indicate that exposure of lung explants and their associated mesenchyme to BMP4 results in stimulation of branching and epithelial cell proliferation (Bragg, Moses, & Serra, 2001). BMP4 is expressed in lung mesenchyme with the highest expression adjacent to the proximal epithelium, including expression in parabronchial smooth muscle (PBSM) cells (Weaver, Batts, & Hogan, 2003). Possibly, the mechanism of BMP4 regulating lung branching is through inducing PBSM precursor cells differentiation into PBSM cell lineage (Mailleux,

Kelly, & Veltmaat, 2005). PBSM cells are found surrounding the proximal air ways (Yamada, Suzuki, Gejyo, & Ushik., 2002), and may participate in embryonic lung growth by a stretch-induced signaling mechanism (Sparrow & Lamb, 2003). Fetal PBSM is spontaneously contractile throughout gestation, resulting in the stretching of the fetal pulmonary epithelium and mesenchyme (Schittny, Miserocchi & Sparrow, 2000). It is possible that the contractility of PBSM may result in the release of growth factors, signaling molecules, or the regulation of gene expression with consequent effects on lung cell growth and differentiation (Nakamura & McCray, 2000).

Bone Morphogenetic Protein 4 and Heart

Gene expression and *in vitro* explant assays suggests that BMP4 is a key myocardial signal in initiating epithelial–mesenchymal transformation (EMT) and driving the formation and/or differentiation of the cardiac cushions (the precursor to the heart valves and immediately adjacent areas). Because of its spatio-temporal patterns of expression, BMP4 is, in fact, thought to play a role in induction and maturation of the cushions in both the OFT and the AVC. Time course study of BMP4 mRNA expression domain within the mouse developing heart tube by *in situ* hybridization found its expression is restricted to the myocardium in cushion-forming segments of the AVC at E9.5. At E10.5, however, BMP4 expression switched to the myocardium of the OFT, becoming absent from the AVC (Jones, Lyons & Hogan, 1991). More recently, the expression domain of BMP4 mRNA has been further investigated and its role in cushion formation confirmed by another study using chick embryos to map the expression pattern of BMP isoforms using *in situ* hybridization (Somi, Buffing, Moorman, & Van Den Hoff,

2004). Somi et al. (2004) found that BMP4 was expressed in the distal myocardial border of the OFT during recruitment of mesodermal cells to the OFT myocardium at HH10-11. As heart development proceeded, BMP4 was expressed in the cardiomyocytes that protruded into the mesenchyme during muscularization of the atrioventricular cushions and the tricuspid valve at HH 33. In addition to studies focused on BMP4 transcripts expression patterns using *in situ* hybridization, the immunolocalization of BMP4 protein expression was also investigated recently. Using polyclonal antibody to immunolocalize the expression domain of BMP4, Keyes et al. (2003) demonstrated that BMP4 was only expressed in myocardia, not in the cushions in both chick and mouse. At chick E5 to E6, for example, BMP4 was expressed restrictly to the myocardium of the OFT. BMP4, however, may not be acting alone. *In vitro* explant assay involving the explantation of heart tube in three dimensional collagen gels indicates that BMP4 by itself is unable to induce cushion formation, suggesting the involvement of additional factors (Mjaatvedt, Lepera, & Markwald, 1987).

Investigation of BMP4 function in cardiac development has been hampered by the early embryonic lethality of BMP4 null mutant embryos (Lawson et al., 1999). Jiao et al. (2003) overcame this difficulty by combining use of a hypomorphic BMP4 allele with a Cre/Loxp approach to manipulate levels of BMP4 expression specifically in cardiomyocytes. Their findings, however, were contrary to previous hypotheses. They showed that BMP4 was dispensable for the initiation of cushion formation but was uniquely required for proper AV septation after cushions have formed. In the developing mouse hearts, even BMP4 was completely deleted, the EMT was normally initiated. In

contrast, after cushions have formed, a modest reduction and a more severe reduction in BMP4 gene expression in mutant mice caused the partial and complete form of AVCD. Nevertheless, the idea that many other BMPs (BMP2, BMP5, BMP6, BMP7, BMP10) are coexpressed in the embryonic heart and may compensate for the loss of BMP4 (Dudley & Robertson, 1997; Lyons, Pelton, & Hogan, 1990; Lyons, Hogan, & Robertson, 1995; Neuhaus, Rosen, & Thies 1999; Somi et al., 2004) compromises their conclusion that BMP4 was dispensable for initiation of cushion formation. Thus gene targeting approaches require double or even triple knockouts to uncover the role of BMP4 during heart development due to the overlapping expression pattern of BMP family.

Another theory as to the possible functions of the BMP4 in the early stage of heart development is that BMP4 is involved in the initiation of an apoptotic molecular cascade in a sub-population of cushion cells (Zhao & Rivkees, 2000; Abdelwahid, Rice, Pelliniemi, & Jokinen, 2001). Existing evidence for an *in vivo* role for BMPs in the induction of apoptosis is inconsistent. BMP4 has previously been shown to induce cell death in tissue culture explants of the endocardial cushions of the OT and AV regions (Zhao et al., 2000). Somi et al. (2004) made this theory more attractive. They found that at the site of delamination, BMP4 mRNA was expressed at interface of myocardium and cushion tissue. During digit formation, BMP4 was found to be essential in the regulation of interdigital cell death (Merino, Ganan, Macias, Rodriguez-Leon, & Hurle 1999; Tang et al., 2000). These observations suggested a role for BMP4 in mediating cell death in delamination of the valve in order to make the valve move freely. However, Allen et al. (2001) failed to find an increase in the levels of apoptosis in the cushions by

overexpressing retroviral noggin (antagonist of BMP4) to inhibit BMP4 signaling. Interestingly, mice deficient for intracellular BMP-signaling molecule, Smad 6 (inhibiting BMP4 signal transduction pathway), developed severe cardiac defects, with hyperplasia of the cardiac valves and defects of septation in the OT (Galvin et al., 2000). Taken together, no unequivocal evidence supports a role for BMP4 in the induction of apoptosis in the developing heart.

Specific Aim

The objective of this research project was to produce anti-chick BMP4 monoclonal antibodies (mAbs) for use in a time-course study of BMP4 expression domains during chick cardiogenesis.

Experimental Design

In order to produce anti-chick BMP4 monoclonal antibodies, two approaches were carried on in parallel: i) Express recombinant chick BMP4 protein (cBMP4) in both prokaryotes and eukaryotes and ii) direct produce monoclonal antibodies using cBMP4 peptides. Primer sets were first designed and tested to allow capture of i) the entire open reading frame of cBMP4 and ii) that portion of the cBMP4 coding region giving rise to the mature (normally proteolytically derived) fragment of cBMP4 (mcBMP4). Second, the successfully amplified PCR products encoding the mature region were purified, linearized and cloned into pcDNA4/HisMax, a mammalian cell expression vector allowing the production of a His tagged BMP4 fusion protein. COS- 7 cells were then transfected with this expression vector under optimized conditions to produce fusion His-mcBMP4 protein. Finally, the His- tagged fusion protein was purified from crude cell

lysate using a nickel-chelating affinity column. Although fusion protein expression was verified by Western Blot in the crude cell lysate, initially weak expression levels coupled with loss suffered during subsequent purification steps resulted in no detectable protein at the final stage of purification. In an alternative approach, an *E.coli* expression system was selected for protein expression and purification. A pGEX-KG vector carrying mcBMP4 insert with the correct reading frame was constructed. *E.coli* BL-21 cells were then transformed with this expression vector under optimized conditions to produce GST fusion protein, GST-mcBMP4. Subsequently, this fusion protein was purified using a GST affinity column and the expression of the fusion protein was verified by western blot.

In the second direct production approach, cBMP4 peptide was first designed using bioinformatics' tools and synthesized by a commercial company. Mice were next immunized following a standard protocol and immunization schedule. Once a specific immune response in immunized mice had been verified, spleenocytes were then harvested and fused with sp2/0 cells for monoclonal antibody production. Clones were subsequently screened for anti-cBMP4 specific monoclonal antibody production.

Materials and Methods

Mature Bone Morphogenetic Protein 4 Expression in Mammalian Cells

Total chick RNA isolation. Two dozen fertilized eggs were bought from Olivera egg ranch (San Jose, CA), incubated for four days at 99±1°F with 100% humidity, and rotated automatically during the incubation in the egg chamber. Incubation times were extended to 4 days to correct for the temporary cessation of development that was found to occur as a result of temperature drop during commercial storage and transportation of

the eggs to the lab. At the end of incubation, chick embryos were harvested surgically, and immediately put into RNAlater solution (Ambion) to inactivate the Rnase activity as rapidly as possible. Net weights of the chicken embryos were obtained in these initial process steps.

Chick total RNA was isolated using reagents supplied in Ambion's ToTALLY RNA™ Total RNA Isolation Kit (Cat#AM1910) and following the manufactures recommended protocol. Chicken embryos were transferred to a 50 ml conical tube containing 10x volume of Denaturing reagent (each mg of tissue represents about 1 µl of volume). Tubes were kept on ice and the tissue homogenized using a mechanical homogenizer and stainless steel rotor. The tissue lysate was collected, poured into screw-capped polypropylene Sorval tubes and centrifuged at 12,000xg for 3-5 min at 4°C to pellet debris. Harvested supernatants were transferred into a 50 ml conical tube. The volume measured was defined as the starting volume. One starting volume of phenol: chloroform: isoamyl alcohol (25:24:1, PH 7.5) was then added to the lysate, the mixture vigorously vortexed for 1 min, incubated on ice for 15 min, and then centrifuged again at 12,000xg for 15 min at 4°C. Following this centrifugation, the upper aqueous phase was harvested, its volume measured, and the solution transferred to a new 50 ml conical tube. An amount of Rnase free sodium acetate (3M, PH 4.5) equal to 1/9th the volume of the transferred aqueous phase was added. The mixture was agitated for 10 seconds to create a homogeneous solution. Next, one starting volume of acid-phenol: chloroform (AP: C) was added to the adjusted aqueous phase. The solution was vortexed for 1 min, stored on ice for 15 min and then centrifuged at 12,000xg for 15 min at 4°C. The aqueous phase

was transferred to a new RNase-free polypropylene tube (with capacity at least twice the volume of this harvested aqueous phase) and the total RNA then precipitated by adding an equal volume of isopropanol with subsequent mixing. The RNA was allowed to precipitate overnight -20°C. After the incubation was complete, the preparation was centrifuged at 12,000xg for 15 min at room temperature. The supernatant was decanted off and the residual salts was removed by gently vortexing (washing) the pellet using freshly made 70% ethanol. RNA was recovered by centrifugation for 5-10 min at 3000xg at room temperature. The final RNA pellets were then resuspended in the desired volume of DEPC water containing 0.1mM EDTA (100 µl per 100 mg of starting material) and the material stored at -80°C until needed (White, 2004).

PCR amplification of chick mature cBMP4. The entire coding region of cBMP4 was amplified using gene specific primers according to the published cBMP4 sequence from GenBank (Accession number: X75915). Complete complementary primers were designed for blunt end ligation using Vector NTI (Invitrogen). Sense primer was 5' ATGATTCCTGGTAACCGAATGC3'; Antisense primer was 5'AGGGAAGGGGTCAGCG 3'. The sense primer started at nucleotide #1 while the antisense primer started at nucleotide #1228. The total number of nucleotides amplified was 1228 bp. RT-PCR was employed using an OneStep RT-PCR kit from Qiagen (Cat# 210210) to generate ds cDNA from chick total RNA under the standard conditions.

RT-PCR reaction conditions were as follows: 1x RT-PCR reaction buffer (containing 15mM MgCl₂), dNTP mix 0.2 mM each, primer 0.6 µM each, 1.3 µg chick total RNA and 1 unit of enzyme mix in a 50 µl reaction volume. RT-PCR amplification

was carried out at 50°C for 30 min, 95°C for 15 min followed by 40 cycles of 94°C for 30 Sec, 65.9°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

The mature region of cBMP4 was amplified using gene specific primers (complete complementary) designed for blunt end ligation using Vector NTI (Invitrogen). Sense primer was 5'AGCCCCAAGCACCACGGTTC3'; Antisense primer was 5'GGGAAGGGGTCAGCGGCAC3'. The sense primer started at nucleotide #874, while the antisense primer started at nucleotide #1219. The total number of nucleotides amplified was 354 bp. Standard PCR was employed to amplify the mcBMP4 using the full length cBMP4 PCR product as template. Taq PCR Core Kit from Qiagen (Cat# 201223) was used to set up PCR reaction following manufactures instructions.

PCR reaction conditions were as follows: 1x PCR reaction buffer (containing 15 mM MgCl₂), dNTP mix 0.2 mM each, primer 0.5 µM each, 100 ng cBMP4 PCR product and 1 unit of Taq polymerase in a 50 µl reaction volume. PCR amplification was carried out at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

Subcloning of mature cBMP4 gene into a mammalian cell expression vector. The pcDNA4/HisMax mammalian cell expression vector from Invitrogen (Cat # V864-20) was selected to produce recombinant mcBMP4. Major features of this vector includes a CMV promoter for high level expression in a wide range of mammalian cells, a QBI SP163 translational enhancer for increased levels of recombinant protein expression, an N-terminal peptide encoding the XpressTM epitope, a polyhisidine metal-binding tag, and an enterokinase recognition site for tag removal.

The expression vector was first linearized using *KpnI* restriction enzyme and repurified. The linearized vector was next given blunt ends by treatment with T4 DNA polymerase (New England Biolab, cat# M203S) and reaction buffer at 12°C for 20 minutes. The blunt end reaction contained 1x T4 DNA polymerase buffer, 100 µg/ml BSA, 0.1 mM each dNTP mix, 0.3 unit of T4 DNA polymerase and 1 pmole of *KpnI* digested vector in a 50 µl reaction. The digested vector was then treated with Aquatic Phosphatase (New England Biolab) for 15 minutes at 37°C to generate dephosphorylated ends. The dephosphorylation reaction contained 1x aquatic buffer, 1 unit of Aquatic Phosphatase and the previously end-converted vector in a 50µl reaction.

The amplified mature cBMP4 PCR product was ligated into the linearized and dephosphorylated blunt ended expression vector. The ligation reactions were performed according to Novagen's protocol described in pST Blue Perfectly Blunt Cloning kit (Cat# 70191). The plasmid constructs were transformed into NovaBlue Singles competent cells (part of Perfectly Blunt Cloning kit) according to the manufacturer's protocol. Cells were plated into IPTG (0.5mM)/Xgal (80 µg/ml)/Amp (100 µg/ml)/LA plates, grown overnight at 37°C, and white colonies were picked and subcultured into a gridded LA plate and incubated overnight. Next day, 10 colonies were picked to perform colony PCR for the purpose of identifying the colonies carrying the desired cBMP4 insert. Colony PCR reaction conditions were as follows: 1x PCR reaction buffer (containing 15 mM MgCl₂), dNTP mix 0.3 mM each, primer 1.5 µM each, 10µl cell lysate and 1 unit of Taq polymerase in a 25 µl reaction volume. PCR amplification was carried out at 94°C

for 3 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

A colony carrying the mature cBMP4 insert was selected, cultured using LB broth with 50 µg/ml Ampicillin, and grown with shaking at 225 rpm overnight at 37°C. Plasmid DNA was isolated the following day according to Qiagen's minielute kit protocol. Plasmid concentration was determined using Nanodrop and sent out to Gene Gateway (Hayward, CA) for DNA sequencing using both T7 (flanking the insert upstream) and BGH primers (flanking the insert downstream) to confirm both the orientation and nature of the insert.

Culture of COS-7 cells for transfection. COS-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The complete medium used for culturing the cells was DMEM supplemented with L-glutamine, penicillin-streptomycin, and 10% heat-inactivated fetal calf serum (FCS). A total of 2×10^5 cells were seeded in a Falcon 25 cm² tissue culture flask with 12 ml culture medium, and the cells were incubated in a 37 °C incubator with 5% CO₂ and 99% humidity. The cells were examined daily using inverted microscope to ensure healthy growth and no contamination. Every 2-3 days, cells were subcultured at the ratio of 1:4 by standard trypsinization (White, 2004). The old medium was removed followed by rinsing the cells with pre-warmed PBS. Then the adherent cells were detached from the bottom of the flask by 2 ml 0.2% (W/V) trypsin-EDTA (1 mM) treatment about 5 min at 37°C. After the cells were detached, 6 ml of complete medium were added to inactivate the trypsin. Four new flasks containing 7 ml of the fresh medium were prepared. A 2 ml

volume of cell suspension was transferred to each new flask, and incubated in the CO₂ incubator.

COS-7 cells transfection condition optimization. In order to access the transfection efficiency, pcDNA/HisMax/lacZ plasmid from Invitrogen (part of pcDNA4/HisMax mammalian cell expression vector) was used as positive control to optimize the transfection condition based on the β -galactosidase expression. Mock-transfected cells in absence of any DNA were also used as negative control to provide protein background.

COS-7 cells were transiently transfected using lipofectamine 2000 reagent (Invitrogen) following the manufacture instructions. One day before transfection, cells were passaged as described earlier and counted using Trypan Blue dye and a hemocytometer. A total of 5×10^5 cells in 2 ml growth medium without antibiotics was seeded on a cover slip (10cm² surface area) placed in a 6- well plate so that cells would be 90-95% confluent at the time of transfection. The medium used for transfection was Opti-MEM I Reduced Serum Medium (Invitrogen), with L-glutamine added to Opti-MEM I prior to use. Antibiotics and serum lower the transfection efficiency by either disturbing the cells or interfering with the liposome mediated mechanism of DNA transfer. Therefore, an hour before transfection, complete medium was removed, and the cells rinsed with CMF-PBS to remove the trace of serum-containing medium. Finally, a total of 2 ml Opti-MEM I was added to each well, and the incubation was continued.

For each transfection sample, the complex was prepared using a DNA mass to Lipofectamine 2000 volume ratio of 1 (μ g): 2.5(μ l) or 1 (μ g): 3 (μ l) to optimize the

transfection. For a DNA to Lipofectamine ratio of 1:2.5, DNA sample was prepared by diluting 4 µg of pcDNA/HisMax/lacZ into 250 µl of Opti-MEM I medium. For a DNA to Lipofectamine ratio of 1:3, DNA sample was prepared by diluting 3 µg of pcDNA/HisMax into 250 µl of Opti-MEM I medium. In another tube, 10 µl lipofectamine reagent were mixed with 240 µl of Opti-MEM I for 1:2.5 DNA to lipofectamine ratio. Nine microliter of Lipofectamine reagent was mixed with 240 µl of Opti-MEM I for 1:2.5 DNA to lipofectamine ratio. After 5 minutes incubation of lipofectamine-Opti-MEM I reagent, the lipofectamine and DNA solution were combined, mixed gently and incubated for 20 minutes at room temperature to allow for the formation of liposome-DNA complexes. Finally, 500 µl Opti-MEM I was removed from each well, and replaced by 500 µl of lipofectamine-DNA complex solution. The plate was returned to incubator to incubate for 4-6 hours. Following incubation, the medium was removed totally and complete medium were added. Cells were incubated continuously and observed for transient expression over 5 days. In addition to a positive control, mock- transfected cells were also used as a negative control.

Staining cells for β -galactosidase activity. At the end of incubation, cells were rinsed twice with sterile CMF-PBS, then fixed by adding 2 ml glutaraldehyde solution (0.25% v/v in CMF-PBS) and incubated at room temperature for 15 minutes. Cells were rinsed again three times with 5 ml CMF-PBS to remove residual glutaraldehyde. Finally, 2 ml X-Gal Working Solution (0.2% w/v X-Gal in CMF-PBS supplemented with 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ and 5 mM $K_3Fe(CN)_6$ was added to each well and incubated 20 minutes. At the end of the staining period, X-Gal solution was removed and

CMF-PBS was added for better viewing of the stained cells. Stained cells were observed under the inverted microscope and images recorded using a Nikon model digital camera.

Preparation of cell extracts. At 48 hour post transfection, cells were rinsed with PBS and harvested in 1 ml PBS using a rubber cell scraper. The harvested cells were transferred into a 15 ml conical tube and centrifuged at 1500xg for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 2 ml cell lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl at PH 7.8. A mammalian tissue protease inhibitor cocktail containing AEBSF, Aprotinin, Bestatin hydrochloride, E-64, EDTA and Leupeptin hemisulfate salt (Sigma) was also added to the cell suspension to prevent protein degradation by endogenous enzymes. The cell suspension was incubated on ice for 15 min. Following the incubation, the cells were further lysed by two freeze-thaw cycles using liquid nitrogen and 42°C water bath. Cells were then sonicated using Sonifier Cell Disruptor model 350, set at 50% duty cycle and power output setting of 5.5 for 5 cycles with 20 s burst and 40 s cooling period for each cycle. After lysis, the cell lysate was centrifuged at 1500xg for 10 min. Then the supernatant containing cell extract was collected and stored at -80°C.

Protein purification using ProBond™ purification system. The fusion protein had six tandem histidine residues on its N- terminus, and this polyhistidine tag has a high affinity for a resin containing nickel ions. Therefore, when the tagged protein was applied to a pre-filled ProBond™ column with ProBond™ nickel-chelating Resin (Invitrogen, Cat# K850-01), it was captured by the column's nickel-chelating resin. The fusion protein was later eluted with an imidazole gradient under native conditions.

Imidazole has a similar structure to that of histidine and can thus desorb tagged protein from the column by competitive substitution. All the buffers used in the system had a pH of 8.

The purification of the His-mcBMP4 fusion protein was performed according to the manufacturer's protocol. In brief, 2 ml of nickel-chelating resin was poured into a 10 ml purification column and allowed to settle completely by gravity. The supernatant was gently removed by aspiration and the column pre-equilibrated by washing twice with 6 ml sterile distilled water and three times with 6 ml Native Binding Buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 8). Prior to loading, 5ml of the concentrated cell extract was raised to 8 ml by adding Native Binding Buffer. Following this dilution step, the sample was applied to the column and mixed with the resin for 60 min at room temperature with gentle agitation to keep the resin suspended in the lysate solution. After the 60 min incubation, the column was washed twice with 8 ml Native Binding Buffer and three times with 8 ml Native Wash Buffer (20 mM imidazole, 20 mM sodium phosphate and 500 mM sodium chloride, pH 6) to remove non-binding and/or low affinity non-specifically bound protein. Following this washing step, the fusion protein was eluted with 10 ml Native elution buffer with an imidazole gradient. The gradient was generated by applying four elution buffers (pH6) in increasing imidazole concentration (50 mM, 200 mM, 300 mM, and 500 mM) into the column. The eluted fraction was collected from the column and each fraction was analyzed with SDS-PAGE.

Western blot analysis. A 4-12% Bis-Tris gradient NuPage minigel (Invitrogen) was used to verify the efficiency of protein purification. A western blot was then used to

verify the presence of recombinant His- mcBMP4 fusion protein. Each sample was diluted using 4x sample buffer (Invitrogen) and heated at 70°C for 10 min. The samples were cooled and quickly centrifuged to remove insoluble debris and loaded into each well of the gel. MES-SDS buffer (50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA pH 7.3) was used as the running buffer. The gel was run at a constant 200 V for 35 min. Following electrophoresis, the proteins separated in the SDS-PAGE gel were electrophoretically transferred to a PVDF membrane (pre-wet in methanol). The XCell II blot Module apparatus was assembled according to manufacturing protocol and the transfer was conducted at a constant 30 V for 1.5 hour using NuPAGE transfer buffer (25 mM bicine, 25 mM bis-Tris, 1 mM EDTA pH 7.2). After the transfer, the gel was exposed to Coomassie blue stain for overnight to evaluate the transfer efficiency. The membrane was stained in Ponceau S solution containing 0.5% (w/v) Ponceau S and 1% (v/v) glacial acid for 10 min, and then rinsed with DI water.

To develop the immunoblot, the PVDF membrane was placed in blocking buffer (pH 7.4) for 1 hour at room temperature. The blocking buffer was prepared by adding 2 g of BSA to a liter of PBS containing 0.05% Tween 20. The membrane was then incubated with primary antibody (anti-Xpress antibody, a mouse monoclonal IgG from Invitrogen) diluted 1:5000 in blocking buffer at 37°C for 2 hours. The primary antibody directed against the Xpress epitope was removed after one hour, and the blot washed with blocking buffer 5 times over 30 min. After washing, the membrane was incubated with the secondary antibody (rabbit anti-mouse IgG-AP conjugate, Sigma) diluted 1:5000 in blocking buffer for 1 hour at room temperature. After incubation, the membrane was

washed 3 times with blocking buffer and twice with PBS/Tween 20 over 30 min. Finally, the membrane was rinsed with water and developed using the BCIP/NBT-substrate (SIGMA FASTA BCIP/NBT Cat# B5655) for 5 min. After developing, the membrane was washed in DI water and then air-dried.

Mature cBMP4 Protein Expression in E.coli

Subcloning mature cBMP4 into a bacterial expression vector. The mature region of cBMP-4 was also amplified using gene specific primers designed by Vector NTI software (Invitrogen). Sense primer was 5' CGAAGGATCCAGCCCCAAGCACACGGTTCCC 3'. Antisense primer was 5' AACAAAGGATCCGTCAGCGGCACCCGCACCCCTC 3'. The sense primer started at nucleotide #874, while the antisense primer started at nucleotide #1219. The total number of nucleotides amplified was 370 bp. Standard PCR was employed to amplify the mature cBMP4 using the full length cBMP4 PCR product by Qiagen's Taq core kit. PCR reaction conditions were as follows: 1x PCR reaction buffer (containing 15 mM MgCl₂), dNTP mix 0.2 mM each, primer 0.5 μM each, 100 ng cBMP4 PCR product and 1 unit of enzyme mix in a 50 μl volume. RT-PCR amplification was carried out at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 67.5°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

The expression vector pGEX-KG was kindly provided by Dr. Soto. Major features of this vector include a Glutathione S-transferase (GST) fusion tag and a thrombin cleavage site. Since GST is highly soluble, recombinant protein fused with GST tag could increase solubility. In addition, GST processes enzymatic activity. The

level of fusion protein expression could be estimated by detecting GST activity.

Thrombin cleavage site is located right after GST for the convenience of tag removal.

The vector was digested using *BamHI*, then dephosphorylated using Aquatic phosphatase to prevent vector religation. The mcBMP4 PCR product, also digested using *BamHI*, was then ligated to the vector according to Novagen's protocol described in pST Blue Perfectly Blunt Cloning kit, and transformed into *E.coli* (BL-21) competent cells (Stratagene) according to manufacturer's protocol. Cells were plated into LA /Amp (100 µg/ml) plates and grown overnight at 37 °C. The next day, 10 colonies were picked to perform colony PCR for the purpose of identifying the colonies carrying the desired cBMP4 insert. A colony carrying what appeared to be mcBMP4 insert was selected, cultured using LB broth with 50 µg/ml Ampicillin, and grown with shaking at 225 rpm overnight at 37°C. Plasmid DNA was isolated the following day according to Qiagen's minielute kit. Plasmid concentration was determined using Nanodrop and sent out to Sequetech (Mountain view, CA) for DNA sequencing using both pGEX3 and pGEX5 primers.

Screening PGEX recombinant for fusion protein expression. Several colonies of *E.coli* transformed with the pGEX recombinants were picked for inoculation into separate tubes containing 2 ml 2xYTA medium containing 16 g/L trytone, 10 g/L yeast, 5 g/L NaCl and 100 µg/ml Ampicillin. The liquid culture was grown to an OD₆₀₀ of 0.6-0.8 with vigorous agitation at 37°C. Cells were induced for two hours by adding 20 µl 100 mM isopropyl-β-D-thiogalactoside (IPTG) (final concentration 1mM), and the liquid cell culture was then centrifuged at 500xg for 5 min. The cells were spun down and lysed

using sonication in 10s burst with 20s rest. The crude cell lysate was centrifuged for 10 min at 7000xg again to remove insoluble material. The supernatant was harvested and added into 50% slurry of Glutathione Sepharose 4B pre-equilibrated in ice cold PBS (pH 7.3). After 30 min incubation, sample was spun down again at 500xg for 5 min to sediment Glutathione Sepharose 4B with recombinant protein attached. PBS (pH7.3) was used to wash the sample three times. The fusion protein was eluted with elution buffer (pH 8.0) containing 50 mM Tris-HCl, 10 mM reduced Glutathione. The eluted fraction was analyzed by 4-12% pre-casted Novex NuPAGE gel (Invitrogen).

Fusion protein expression optimization. After the candidate expressing recombinant protein was selected, important factors such as growth temperature and induction conditions have been evaluated to achieve optimal protein expression.

A selected candidate colony was subcultured overnight at 37°C. Several colonies were picked and transferred into separate tubes containing 40 ml 2xYTA medium. Cells were cultured either at 30°C or 37°C until its OD₆₀₀ reached about 0.6-0.8. Five different IPTG concentrations (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1 mM) were added into the liquid culture, and the cells were induced for different time period (2 hours, 4 hours, 6 hours, 8 hours and 10 hours). After induction, cells were lysed by sonication as previously indicated. The supernatant was harvested and the relative level of GST fusion protein expression was analyzed by GST activity using CDNB (1-chloro-2, 4-dinitrobenzene) as the substrate. The CDNB solution contains 0.1 mM KH₂PO₄ (pH 6.5), 1mM CDNB and 1mM reduced glutathione. Eight hundred microliter of CDNB was transferred into one UV-transparent cuvettes containing 50 µl of the cell lysate

supernatant and the absorbance was measured at 340 nm at 1 min intervals for 5 min. $A_{340}/\text{min/ml}$ was calculated as a value to indicate relative GST fusion protein content. $\Delta A_{340}/\text{min/ml} = [A_{340}(t_2) - (t_1)] / (t_2 - t_1)$ (ml sample added), where $A_{340}(t_2)$ = absorbance at 340 nm at time t_2 in min. (t_1) = absorbance at 340 nm at time t_1 in min.

Mature cBMP4 protein expression and purification in E.coli. After the expression condition was optimized, 100 ml of cells (2 liter cells for large scale) were grown and induced at the optimized condition. Cell pellets were collected and sonicated as previously described for small scale protein expression in 100 ml. Cells were lysed using Bug Buster reagent (Novagen, Cat# 70584) supplemented with 0.1 mM PMSF solution for large scale protein expression in 2 liter. A crude cell lysate was purified using Glutathione Sepharose 4B beads as described above. Western Blot analysis was employed to verify the expression of fusion protein. The detailed procedures were described in previous section. The primary antibody was rabbit anti- GST polyclonal antibody from GE health care at a 1:1000 dilution. The secondary antibody was goat anti-rabbit IgG-HRP polyclonal antibody from Sigma at a 1:2000 dilution.

Monoclonal Antibody Production Using Synthetic Peptide

Selection of synthetic peptides. The complete sequence of chick BMP4 was obtained from the protein sequence database of the National Center of Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The antigenic region of the mature cBMP4 protein was analyzed using the Protean function (protein structure analysis & prediction) of the Lasergene software from DNASTAR to predict the peptide's secondary structure, hydrophilicity, surface availability and antigenicity. In addition, the amino acid sequence

of cBMP4 was also submitted to Alpha Diagnostic International (San Antonio, TX) for an independent antigenicity analysis.

To ensure that the selected regions were unique to chick BMP4 rather than common family member, the complete mcBMP4 amino acid sequence was compared to the sequence of its closest family member (chick BMP2) and BMP4 sequences in two other species (human and mouse). The amino acid sequences of mouse BMP4, human BMP4, chick BMP4, mouse BMP2, Human BMP2 and chick BMP2 were imported into Biology Workbench (<http://seqtool.sdsc.edu/CGI/BW/cgi>) from the protein sequence database. One peptide sequence SPKHHGSRKNKKNCRRH was selected for synthesis.

Manufacture of synthetic peptides. Chick BMP4 peptide was manufactured by Alpha Diagnostic International (San Antonio, TX). Ten milligram of cBMP4 peptide (average mass: 2071.0 g/mole) was synthesized at 100% purity. Six milligram of cBMP4 peptide was coupled to 3 mg of keyhole limpet hemocyanin (KLH) using glutaraldehyde (GLUT) coupling method. A cBMP4 –KLH conjugate was supplied as 2 mg/ ml solution in PBS and stored in 0.3 ml aliquots at -70 °C. The remaining 4 mg of unconjugated cBMP4 peptide was supplied in a powder form and stored at -70 °C.

Animals. Female BALB/C mice were purchased and taken cared by University Animal Care (UAC) Personnel according to approved guidelines in IACUC (Institutional Animal Care and Use Committee) protocol No.867 (Appendix 1).

Mice immunization for screening assay. Three mice were immunized with cBMP4-KLH conjugated peptides following the immunization schedule (Table 1). In brief, one hundred microgram conjugated peptides in complete Freund's adjuvant (CFA)

(0.3 ml) from Sigma were injected into each mouse via Intraperitoneal (IP) routes for the first immunization. One hundred microgram conjugated peptides in incomplete Freund's adjuvant (IFA) (0.2 ml) from Sigma were injected into each mouse subcutaneously (SC) for the second immunization. One hundred microgram conjugated peptides only (0.2 ml) were injected into each mouse subcutaneously for the third and fourth immunization.

Blood samples were collected as indicated by UAC personnel. Blood samples were combined and allowed to stand at room temperature for 20 min to allow the clot to form, and then samples were centrifuged for 10 min at 12,000rpm to prepare serum. After the immune response has been verified by ELISA, mice were primed with 0.1 ml pristine 3 days before injection of SP2/0 mouse myeloma cells. Then 1×10^6 SP2/0 cells (in 0.2 ml PBS) were injected into mouse abdomen via IP route. A week later, mice ascites were harvested. Mice which exhibited moderate abdominal distention, lack of appetite, pale skin and an increase of respiratory effort were induced to narcosis using carbon dioxide and cervically dislocated under supervision of ACF staff. Ascites was withdrawn using an 18 gauge needle post-mortem by peritoneal cavity tapping. Ascites samples from three mice were pooled together for optimizing the screening assay.

Screening assay optimization and antibody specificity analysis by ELISA.

Screening of specific antibody producing hybridomas is a very critical step since the success of this project is largely dependent on a reliable screening assay for estimating the immune response of the mice and selecting antibody producing hybridoma cells. The amount of antigen required to coat each plate and the dilution of primary immune serum and secondary antibody thus needed to be optimized.

The specificity of the antiserum was analyzed by ELISA using the following protocol. Polystyrene 96-well plates (Nalgene Nunc International, Rochester) were coated with cBMP4 peptides in PBS solution (10µg/ml) overnight at room temperature. The next day, the peptide solution was discarded and the blocking buffer containing 10% skim milk in water was added into each well. The plate was blocked at room temperature for one hour. After 1 hour blocking reaction, the plate was washed three times using PBS with 0.05% Tween. Half of the plate was then incubated with 100 µl of different dilutions of immune or pre-immune sera for one hour at room temperature, while the other half of the plate was incubated with 100 µl of different dilutions of immune sera pre-absorbed with 500 ng antigenic cBMP4 peptide. After incubation, wells were washed again three times and treated with 100 µl horse radish peroxidase (HRP) conjugated rabbit anti-mouse IgG (Sigma) at 1:500 dilution for one hour at room temperature. Next, the wells were washed five times and incubated with 0.1 M 3, 3', 5, 5'-Tetramethylbenzidine (TMB) (Sigma). The absorbance value at 405 nm was then measured using an automatic ELISA plate reader.

Table 1. Mice immunization schedule for screening assay

Week	Procedure
1	Preimmune blood sample 1 ^o antigen/ adjuvant injection (1:1 antigen in CFA, 0.3 ml IP)
3	2 ^o antigen/ adjuvant injection (1:1 antigen in IFA, 0.2 ml SC)
5	3 ^o antigen/ adjuvant injection (antigen in saline, 0.2 ml SC)
7	4 ^o antigen/ adjuvant injection (antigen in saline, 0.2 ml SC) Immune blood sample collection

Cell fusion for monoclonal antibody production. After the specificity of the mice immune response to cBMP4 peptides were verified by ELISA, three mice were immunized again following the protocol indicated above. After the immune response has been verified by ELISA, two mice showing the strongest immune response were sacrificed by cervical dislocation, and their spleens were harvested in a sterile manner. The spleen was gently dissociated mechanically by pressing the tissue against a sterile nylon screen and the spleenocytes were isolated by layering the cell suspension onto 10 ml Ficoll density medium. Cells were then centrifuged cells at 600xg for 20 min in Ficoll density medium. Approximately 1×10^7 of the isolated spleenocytes were fused *with* 1×10^7 SP2/0 cells in 50% polyethylene glycol (PEG) fusion medium slowly. The fused cells were then transferred into hypoxanthine aminopterin thymidine (HAT) medium and aliquoted into several 96-well tissue culture plates and incubated at 37° C. Hybridomas were screened one week after cell fusion by ELISA.

Results

Bone Morphogenetic Protein 4 Expression in Mammalian Cells

Total chick RNA isolation. Total RNA was isolated from chick embryos using Ambion's Total RNA system. The concentration of total RNA was determined by measuring RNA's absorbance at 260 nm (OD260 nm). The purity of total RNA was also estimated by measuring the ratio of UV absorbance at 260& 280 nm. Finally, the integrity of total RNA sample was evaluated by 2% agarose gel electrophoresis. From 12 chick embryos (about 1.5 g), 9.2 mg total RNA (680 µl) was isolated with a concentration of 13.67 µg/µl. From gel analysis (Figure 1), the total chick RNA was

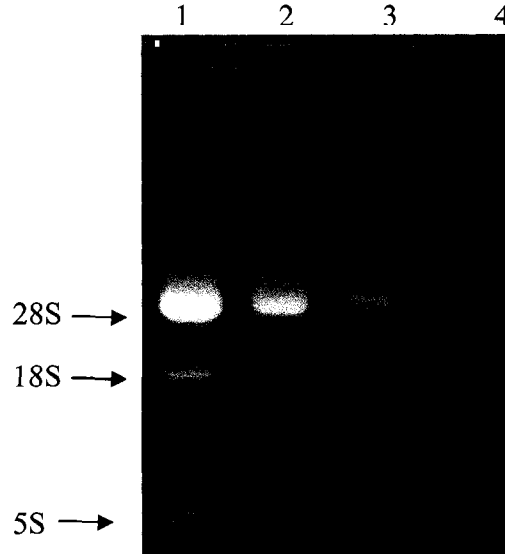


Figure 1. Analysis of different amounts of total RNA isolated from day 4 chick embryos using 2% agarose gel electrophoresis. Samples were run in TAE buffer at 100V for 60 min using 2% of E- gel. Lane 1= 3 μ g total RNA; Lane 2= 2 μ g total RNA; Lane 3= 1 μ g total RNA. Lane 4= 1kb MW ladder.

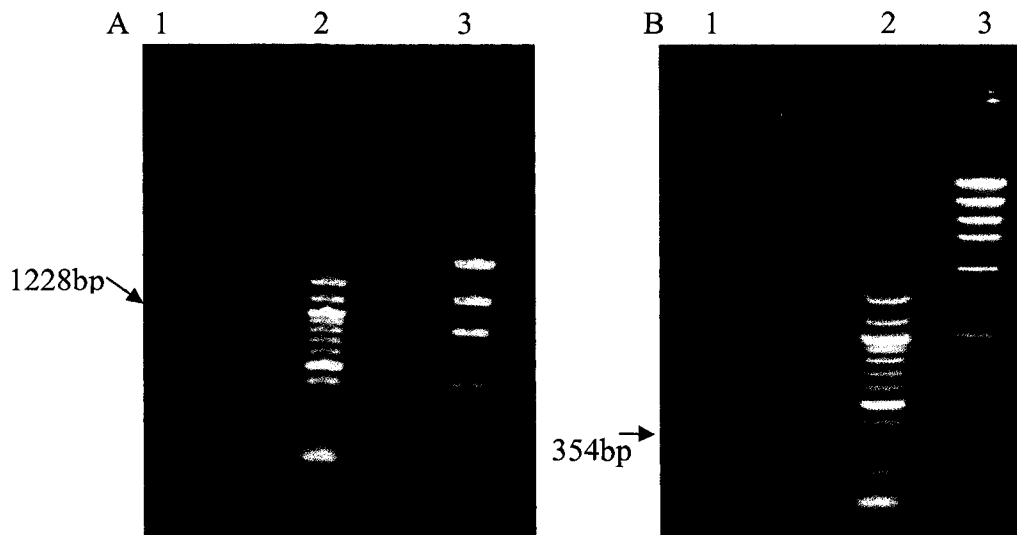


Figure 2. Gel Analysis of cBMP4 inserts amplified from chick total RNA using PCR. Samples were run in TAE buffer at 100V for 60 min using a 1% agarose gel. Panel A shows the amplified cBMP4 insert containing the entire open reading frame. Lane 1= 1228 bp cBMP4 insert containing the entire open reading frame; Lane 2= 100bp size ladder; Lane 3= High MW mass standard. Panel B shows the amplified cBMP4 insert containing only the mature region of the cBMP4 DNA sequence. Lane 1= 354 bp mcBMP4 insert containing the mature region (874bp-1227 bp) of the cBMP4 sequence; Lane 2= 100bp size ladder; Lane 3= Low MW mass standard.

intact since the 28s, 18s and 5s rRNA bands were distinctive without degradation. The RNA sample appeared relatively free of protein contamination since the OD_{260nm}/OD_{280nm} ratio was 2.08.

PCR amplification of cBMP4 entire coding region and mature region. A 1228bp cBMP4 sequence was amplified from chick total RNA using RT-PCR (Figure 2A) and ligated into Novagen's blunt end cloning vector pSTBlue-1 for storage in the long term. In the mean time, mature cBMP4 was also amplified using 1228 bp cBMP4 PCR product as template. The amplified mature cBMP4 insert with 354 bp length containing the region from 874 bp-1227 bp of the cBMP4 sequence was indicated in Figure 2B.

Subcloning of mature cBMP4 gene into a mammalian cell expression vector. The mature cBMP4 inserts were ligated into pretreated blunt ended expression vector cDNA4/HisMax. After transformation of the constructed expression vector, cells grown on the LA/AMP plates were selected to perform colony PCR using gene specific primers in order to identify the colonies carrying the desired mcBMP4 insert. Gel analysis of colony PCR products of individual E. coli clones indicated that all clones contained the desired mcBMP4 inserts (Figure 3). In order to confirm the sequence and orientation of the cBMP4 inserts, plasmids were isolated from three clones and sent out for DNA sequencing.

A Blast search within the Genbank NCBI database confirmed the identity of the inserted DNA sequence to be chick mature BMP4 and the mcBMP4 was ligated in the correct orientation with the pcDNA/HisMax vector (Appendix 2).

The partial DNA sequencing chromatogram of the pcDNA/HisMax-mcBMP4 construct contained the first 24 bp of mature chick BMP4 sequence from nucleotide 287 to nucleotide 310 (red underline) that ligated in frame with the enterokinase recognition sites (yellow highlighted region). The arrow indicates the junction site between mcBMP4 sequence and vector enterokinase recognition sequence, which further indicated that the recombinant protein has the correct reading frame and orientation. The DNA sequence (nucleotide 263 to nucleotide 286) underscored with blue dash line was the Xpress Epitope, which can be recognized by anti-Xpress antibody to facilitate recombinant protein identification (Figure 4). The full pcDNA/HisMax-mcBMP4 construct DNA sequencing chromatogram was indicated in the Appendix 3.

COS-7 cells transient expression condition optimization. COS-7 cells were transfected transiently using pcDNA/HisMax/LacZ control vector at two different Lipofectimin/DNA ratios (1:2.5 vs 1:3). In addition, the level of β -galactosidase protein expression was estimated using X-gal staining for five continuous days (Figure 5). β -galactosidase expression reached about 30% after 24 hour of transfection (Figure 5 A), and then the maximum level of expression was achieved after 48 hours of transfection, which was about 95% cells expressing β -galactosidase (Figure 5 B). From day 3, protein expression level decreased dramatically to about 20% (Figure 5 C), and almost disappeared after day 4 (Figure 5 D-E). β -galactosidase expression was not detected in mock transfected cells (Figure 5 F). Because of the decline of protein expression after 48 hours, transient expression condition at two different Lipofectmine/DNA ratios was optimized only at day two. There is no remarkable difference in terms of protein

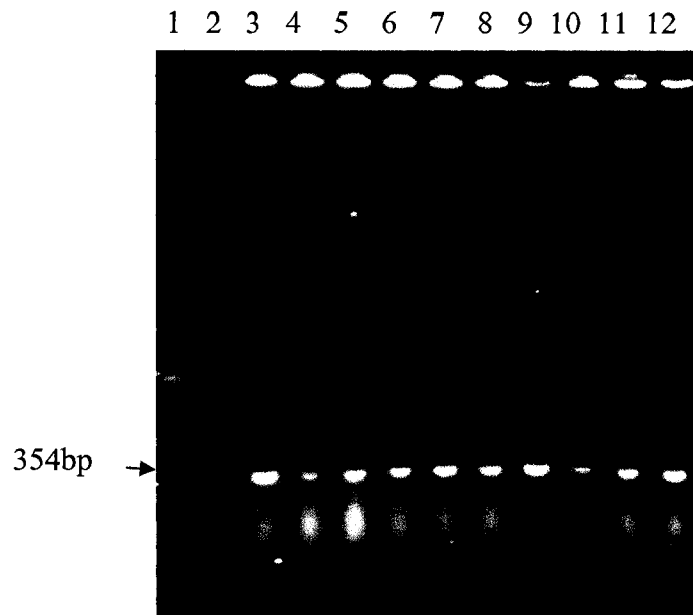


Figure 3. Gel analysis of colony PCR products of individual *E. coli* clones carrying the mcBMP4 insert. All clones contained the desired inserts. pcDNA4/HisMax expression vector was used to clone mcBMP4 gene for mcBMP4 expression in COS-7 cells. Colonies were boiled for 10 min and 10 μ l cell lysate were used for colony PCR. Lane 1= 100 bp DNA ladder; Lane3-12 = PCR products of selected individual *E. coli* clones.

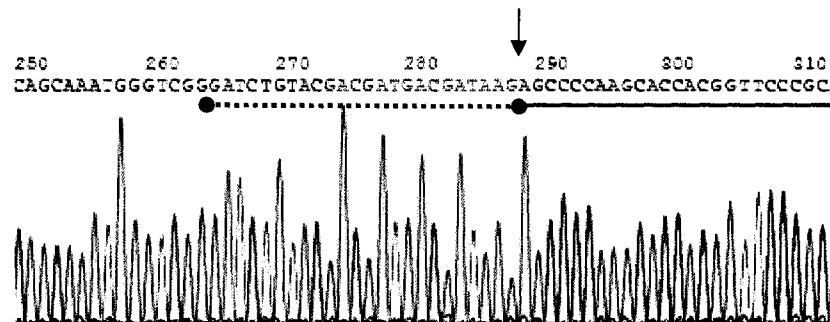


Figure 4. Partial DNA sequencing chromatogram of pcDNA4/HisMax-mcBMP4 construct. Red underline: mcBMP4 DNA sequence; Yellow highlight: EK recognition site; Blue dash line: Xpress epitope. Arrow indicates the ligation site of mcBMP4 insert with the pcDNA4/HisMax vector sequence.

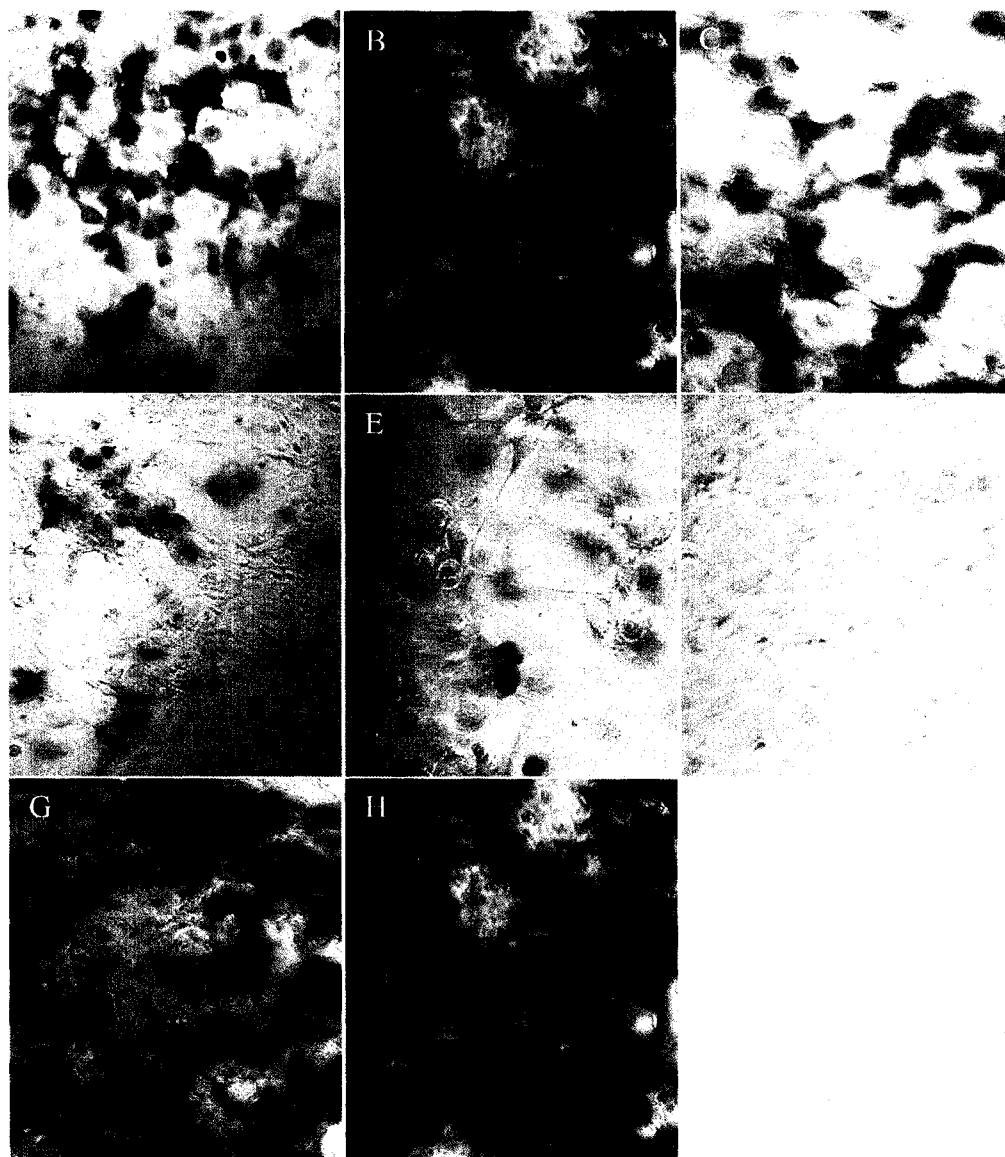


Figure 5. X-Gal staining of COS-7 cells to optimize transient expression conditions. COS-7 cells were transfected transiently using pcDNAHisMax/LacZ control vector and different Lipofectamine/DNA ratios. β -gal expression was monitored over 5 days. Panel A-E: cells expressing β -gal from day 1 to day 5 post-transfection using a Lipofectamine/DNA ratio of 3:1; Panel F: mock transfected cells without β -gal expression. Panel G-H: Comparison of transient transfection efficiency using two different Lipofectamine/DNA ratios at day 2. Lipofectamine/DNA =2.5:1(Panel G). Lipofectamine/DNA =3:1 (Panel H).

expression level by varying lipofectamine vs DNA ratio (Figure 5 G-H).

Lipofectamine/DNA ratio of 2.5:1 was chosen to conduct transfection using constructed expression vector pCDNA4/HisMax_mcBMP4.

Mature cBMP4 protein expression and purification. After the transient transfection condition had been optimized and the transfection efficiency verified by the β -gal staining assay, Cos-7 cells was transfected with the pcDNAhismax-mcBMP4 plasmid. Crude cell lysate was purified using the ProBondTM purification system, and each elution fraction was examined for the presence of recombinant protein via Western Blot (Figure 6). The predicted MW of mcBMP4 is about 12.8 KD, while the N terminal peptide containing the Xpress epitope and the polyhistidine tag will add approximately 3.4 KD to the mcBMP4 protein. The total MW of the recombinant mcBMP4 protein is thus approximately 16.2 KD. The western blot analysis showed that only the crude lysate fraction (lane 1) and the flow-through fraction (lane 11) contained any detectable recombinant mcBMP4 protein. No detectable recombinant protein was present either in the wash fraction (lanes 2-4) or elution fraction (lanes 6-10).

Mature cBMP4 Protein Expression in E.coli

Subcloning of mature cBMP4 into a bacterial expression vector. Mature cBMP4 was also amplified using 1228 bp cBMP4 PCR product as template. The 370 bp mature cBMP4 amplification product contained the region from 874 bp-1219 bp of the cBMP4 sequence and a linker region with BamHI sites (Figure 7). The mature cBMP4 insert was

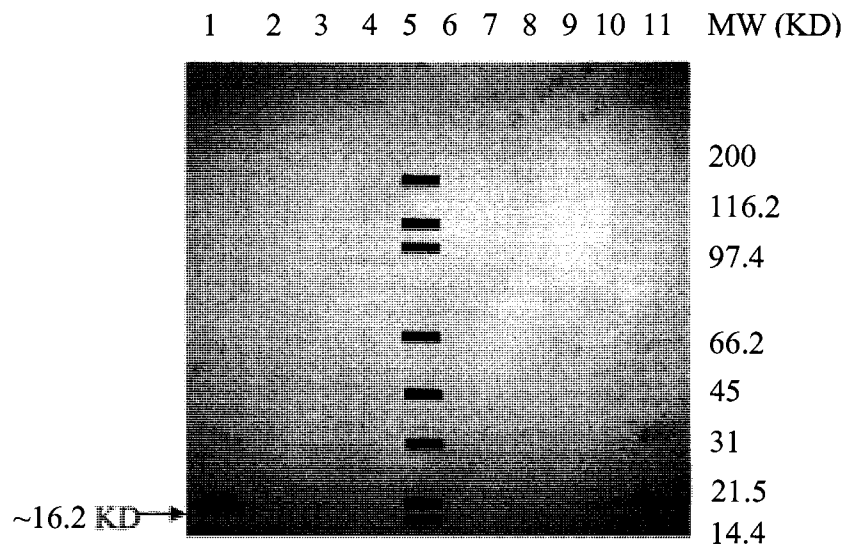


Figure 6. Detection of recombinant mcBMP4 protein expression in COS-7 cells using a 1:5000 dilution of anti-Xpress mAb by western blot. Recombinant mcBMP4 was purified using a nickel chelating affinity column and eluted with different concentrations of imidazole. Lane 1= crude lysate; Lane 2-4= three individual wash fractions. Lane 5= MW standard; Lane 6-10= elution fraction with 50 mM, 200 mM, 300 mM and 500 mM imidazole respectively. Lane 11= flow through fraction.

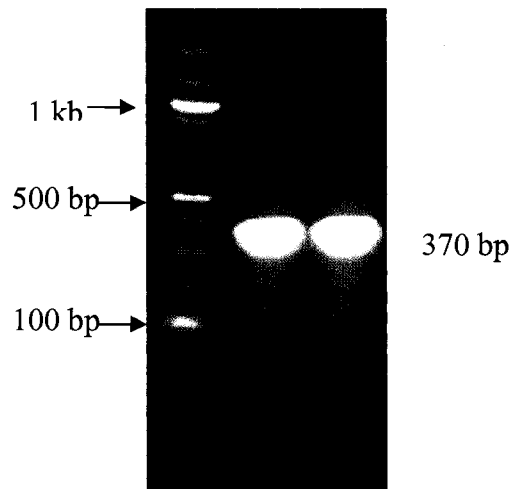


Figure 7. Gel analysis of mcBMP4 inserts amplified by PCR from 1228 bp cBMP4 DNA for cloning in pGEX-KG *E.coli* expression vector. Lane 1= 100 bp DNA ladder; Lane 2 and lane 3= 370 bp amplified mcBMP4 inserts containing *Bam* *HI* site for cloning in pGEX-KG vector.

then ligated into the Bam HI digested E.coli expression vector pGEX-KG. After transformation of the constructed expression vector, cells grown on the LA/AMP plates were selected to perform colony PCR using gene specific primer in order to identify the colonies carrying desirable mcBMP4 insert. From ten randomly selected clones, only one clone (clone 10) contained the 370 bp mcBMP4 insert (Figure 8). In order to confirm the identity and orientation of the mcBMP4 insert, pGEX-KG-mcBMP4 plasmids were isolated and sequenced. Blast search within Genenbank confirmed the identity of the inserted DNA sequence to be mcBMP4 and the mcBMP4 was ligated in the correct orientation with the pGEX-KG vector (Appendix 4). The partial DNA sequencing chromatogram of pGEX-KG-mcBMP4 construct indicated that the first 63 bp of the mature chick BMP4 coding region (from nucleotide 874 to nucleotide 936, red underline) was ligated in frame with the Bam HI linker site (yellow highlighted region) in the pGEX Vector (Figure 9). The full pGEX-KG-mcBMP4 construct DNA sequencing chromatogram was indicated in the Appendix 5.

Screening PGEX recombinants for fusion protein expression. Clone 10 was restreaked and five clones (A1 through A5) were chosen as the potential candidates for GST-mcBMP4 fusion protein screening assay. The predicted MW of mcBMP4 is about 12.8 KD, while the N- terminal peptide containing the GST protein tag and thrombin cleavage site will add approximately 26 KD to the fusion GST-mcBMP4 protein. Thus the predicted total MW of the recombinant mcBMP4 protein is approximately 40 KD.

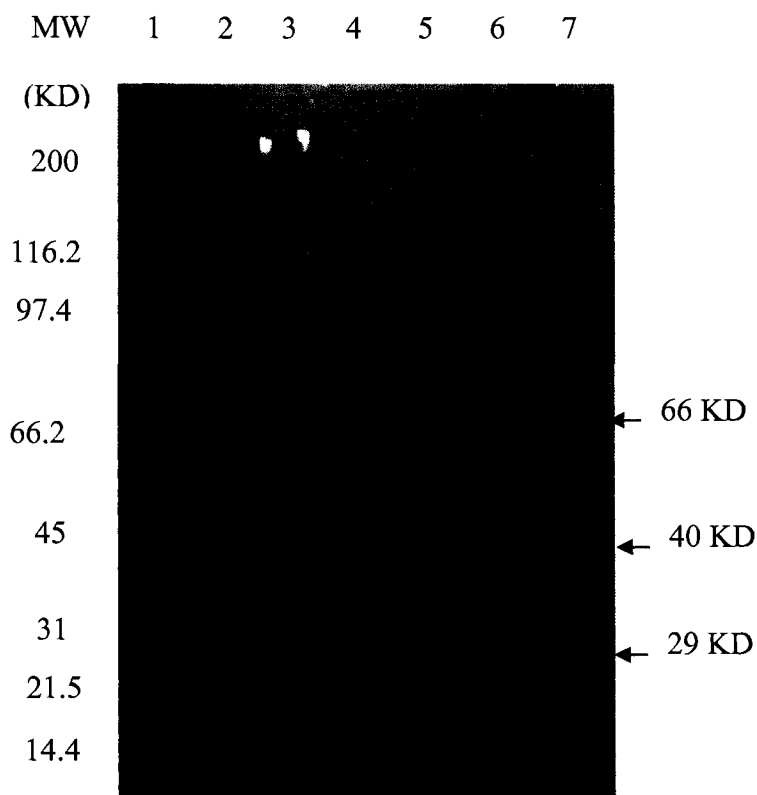


Figure 10. SDS-PAGE analysis of *E.coli* clones in screening for GST-mcBMP4 fusion protein expression. Cells were grown in 2x YTA medium to reach an OD₆₀₀ of 0.6-0.8, and induced using 1mM IPTG for two hours. Crude cell lysate was analyzed using 4-12% Bis-Tris NuPAGE gel in 1x MOPs buffer, running at 200 V for 50 min. The gel was stained overnight using Coomassie blue right after the run. Lane 1=MW standards; Lane 2-6= cell lysate from selected individual clones. Lane 7= cell lysate from pGEX-KG transfected control cells, confirming control GST protein expression.

SDS-PAGE analysis of the five selected clones (lane 2-6) indicated that all five clones contained the 40 KD fusion protein bands (Figure 10). Compare to other clones, clone A4 (lane 4) demonstrated the highest level of protein expression. The control plasmid (expression vector pGEX-KG without mcBMP4 insert) in the last lane produced a strong GST protein band with expected molecular weight of 29 KD (26 KD GST protein plus

3KD thrombin cleavage site). Besides the expected 40 KD fusion protein bands, a band with approximately 66 KD was also observed for all the clones other than control. It is possible that the 66KD band suggests a large conformation change upon dimerization of mcBMP4 to form a tight insoluble or toxic aggregate. Alternatively, it is also possible that some protein interactions present. The fact that more 66KD forms present in most of these clones than the expected 40 KD monomeric fusion protein may suggest that dimerization/aggregation or protein- protein association confers much greater protein stability than is found when the protein is monomeric.

Fusion protein expression optimization. Optimization of the GST-mcBMP4 fusion protein production was investigated by varying IPTG concentration, growth and induction temperature, and induction time. After optimization, a concentration of 1mM IPTG, an induction temperature of 37°C, and induction time of 2 hours were used for subsequent inductions in 2x YTA medium.

Small scale protein purification. Small scale (100 ml) protein expression was conducted under the optimal conditions listed above. GST-mcBMP4 fusion was produced as a monomer with apparent MW around 40 KD. After thrombin cleavage, the expression tag remained but the mcBMP4 was not detectable (Figure 11). Western blotting further confirmed the expression of GST fusion protein using anti-GST polyclonal antibody (Figure 12). In an effort to improve the protein yield, large scale (2 liter) protein expression was also conducted using Bugbuster lysis reagent supplemented with PMSF. No significant improvement of the protein yield was observed (data not

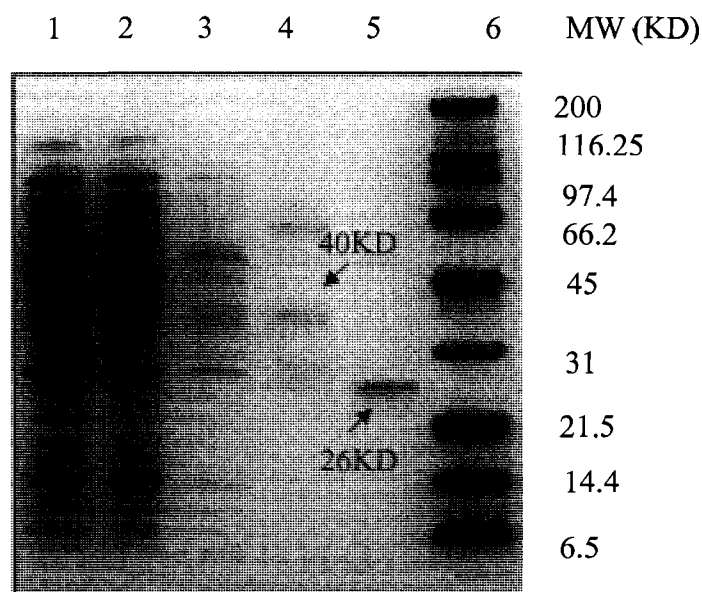


Figure 11. Coomassie-stained 4-12% Bis-Tris NuPAGE gel showing GST-mcBMP4 fusion protein expression in *E. coli* (2 hour induction at 37°C). Crude cell lysate was purified using glutathione sepharose 4B affinity column and eluted with 10 mM reduced glutathione. Gel was run at 200 V for 30 min in 1x MES buffer. Lane 1= crude cell lysate; Lane 2= flow through; Lane 3= pooled wash fraction; Lane 4=eluted fraction; Lane 5= eluted protein with GST tag removed after thrombin treatment. Lane 6= MW STD.

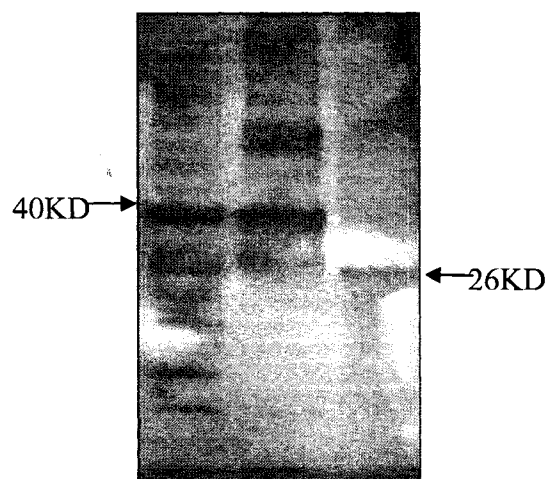


Figure 12. Detection of GST-mcBMP4 fusion protein expression in *E. coli* using 1:1000 dilution of the anti-GST polyclonal antibody by western blot. Lane 1= crude cell lysate; Lane 2= eluted fraction; Lane 3= eluted protein with GST tag removed after thrombin treatment.

shown). The estimated protein concentration was approximately 15 µg/ml. Total protein yield is 300 mg in 2 L scale.

Monoclonal Antibody Production

Peptide selection. The selection of an antigenic peptide is the first and probably the most critical step in the production of a monoclonal antibody. For a synthetic peptide to be sufficiently antigenic, peptide selection should follow several principles. First, the length of the peptide should be 6-25 amino acids long in order to form an immunogenic epitope. It should be part of the mature protein, and mostly composed of hydrophilic residues. Regions of proteins that are mostly composed of polar residues will be more hydrophilic and are more likely to be exposed on the surface of the protein. It should be accessible and not have residues that could be sites of glycosylation. Amino acids buried inside an alpha helix or beta sheet will not elicit antibodies that recognize native protein. Furthermore, for membrane spanning proteins, the peptide should not be within the transmembrane domain, but should be present on the extracellular domain of the protein. Finally, the desired peptide region should be specific and unique, and not have high sequence homology with its close family members. In our project, therefore, only the mature region of cBMP4 with low sequence homology with other BMP proteins was chosen to be analyzed using Protean (DNASTAR, Madison).

The full length cBMP4 protein sequence with its potential glycosylation sites is shown in Appendix 6. Multiple sequence alignment of BMP4 with its closest family member BMP2 indicated that only the N- terminals of the BMP4 protein diverged significantly in sequence homology when compared to BMP2, with the rest of the protein

being highly conserved (Figure 13). It was also evident that the majority of the BMP4 residues at the N-terminals showed high sequence homology across other species, which may indicate that it is likely that antibodies raised against the peptides selected from this region may cross-react with other species.

```

BMP2_Mouse      QAKHK--QRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPPF
BMP2_hm         QAKHK--QRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPPF
BMP2_GG         QAKHK--QRKRHKYSCKRHPLYVDFNDVGWNDWIVAPPGYSAFYCHGECPPF
BMP4_Mouse      SPKHHPPQRSRKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPPF
BMP4_Hm         SPKHHSQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPPF
BMP4-GG         SPKHHG--SRKNKKNCRRHALYVDFSDVGWNDWIVAPPGYQAFYCHGDCPPF
                ..**:::.*:*.*****.*****.*****:***

BMP2_Mouse      LADHLNSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEG
BMP2_hm         LADHLNSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEG
BMP2_GG         LADHLNSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEG
BMP4_Mouse      LADHLNSTNHAIVQTLVNSVNSIPKACCVPTELSAISMLYLDYDKVVLKNYQEMVVEG
BMP4_Hm         LADHLNSTNHAIVQTLVNSVNSIPKACCVPTELSAISMLYLDYDKVVLKNYQEMVVEG
BMP4-GG         LADHLNSTNHAIVQTLVNSVNSIPKACCVPTELSAISMLYLDYDKVVLKNYQEMVVEG
                *****.*****.*****:*****:*****

BMP2_Mouse      CGCR
BMP2_hm         CGCR
BMP2_GG         CGCR
BMP4_Mouse      CGCR
BMP4_Hm         CGCR
BMP4-GG         CGCR
                *****

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Figure 13. Multiple sequence alignment of mature BMP 4 sequence with BMP2 sequence across the species. Blue (*) identical residue. Green (:) structurally conserved residue.

Four aspects of the mcBMP4 peptide sequence were analyzed using the Protean function of the Lasergene software: secondary structure, hydrophilicity, antigenicity and accessibility (Figure 14). The Lasergene software uses the original method of Chou and Fasman to predict helices, sheets, and turns (Chou & Fasman, 1978). The secondary structure of BMP4 was also predicted according to a slightly modified method of Garnier - Robson (Garnier, Osguthorpe, & Robson, 1993). Hydrophilicity is calculated according

to the algorithm of Kyte and Doolittle (Kyte & Doolittle, 1992). They evaluated the hydrophilic and hydrophobic tendencies of the sequence. This profile was useful for predicting exterior vs. interior regions of the native protein. Surface probability was calculated according to the formula of Emini (Emini, Hughes, Perlow, & Boger, 1985).

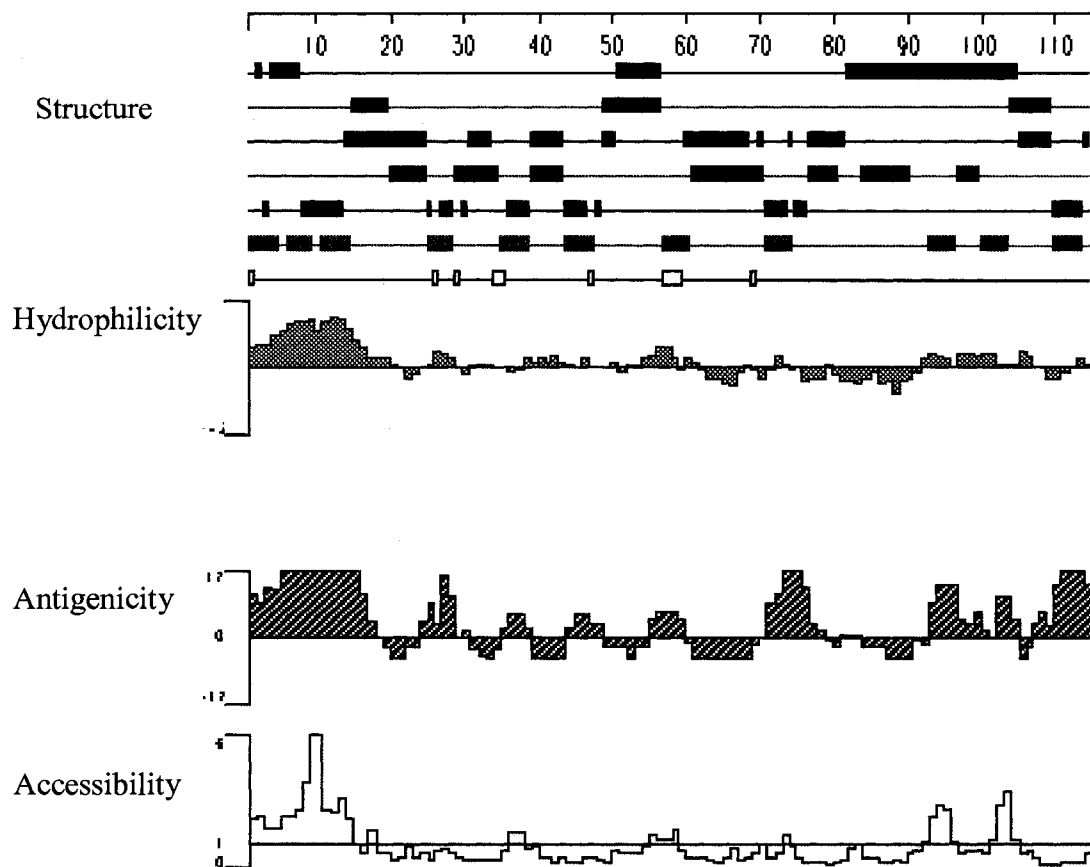


Figure 14. BMP4 antigenic peptide selection using Protean. Four aspects of the peptide sequenced were analyzed: secondary structure, hydrophilicity, antigenicity and accessibility.

The antigenic index (AI) is a measure of the probability that a region is antigenic. It is calculated by summing several weighted measures of secondary structure (Jameson & Wolf, 1988). The outputs of peptide prediction were indicated in Figure 14. The N-terminal sequence (residue 1-20) was predicted to be highly hydrophilic, accessible and antigenic with very little alpha helix or beta sheet structure. Together with the multiple sequence alignment results, one peptide sequence (SPKHHGSRKNKKNCRRH) with a total of 17 residues was selected for peptide synthesis.

Screening assay optimization. The dilution of the primary and secondary antibody was optimized using ELISA for use in a screening assay. The dilution of primary antibody was started as 1:20, and then a series of two fold dilution was made. The dilution of secondary antibody was started at 1:500. In general, the mice immune response to cBMP4-KLH conjugates was very weak and no reaction was detectable when the immune serum's dilution was higher than 1: 40. The absorbance (405 nm) values of the immune serum (red bar) were considerably higher than those of the pre-immune serum (yellow bar) when assayed using a 1: 500 dilution of the secondary antibody (Figure 15). Primary antiserum dilution of 1: 20 and secondary antibody dilution of 1: 500 were chosen to further analyze the immunogenicity of BMP4 peptide.

The amount of peptide (500 ng to 7.8 ng) to coat the ELISA plates was also optimized and evaluated using three pooled immune serum dilutions (Figure 16). The higher the immune serum dilution, the lower the absorbance value was expected. However, the higher the amount of antigen coating the plate, the lower the absorbance intensity was found for each dilution. It seemed that the peptides were present in the

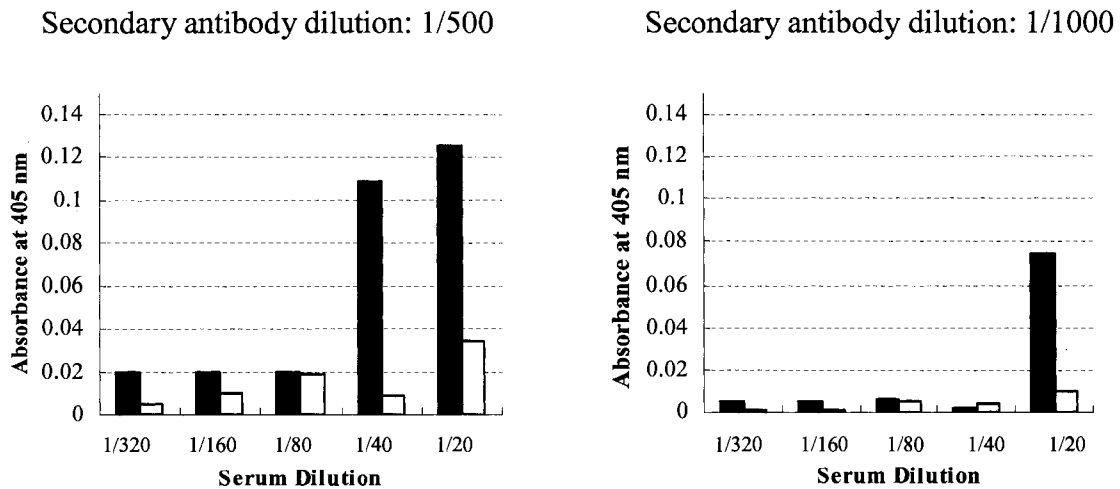


Figure 15. ELISA analysis using different secondary antibody dilutions. Absorbance (405nm) values of anti-cBMP4 immune serum at different secondary antibody dilutions. ELISA plates were coated with 1 μ g synthetic cBMP4 peptide and incubated with serial dilutions of pooled mice immune serum. A serial dilution of the primary Ab was tested at two different HRP conjugated secondary antibody concentrations.

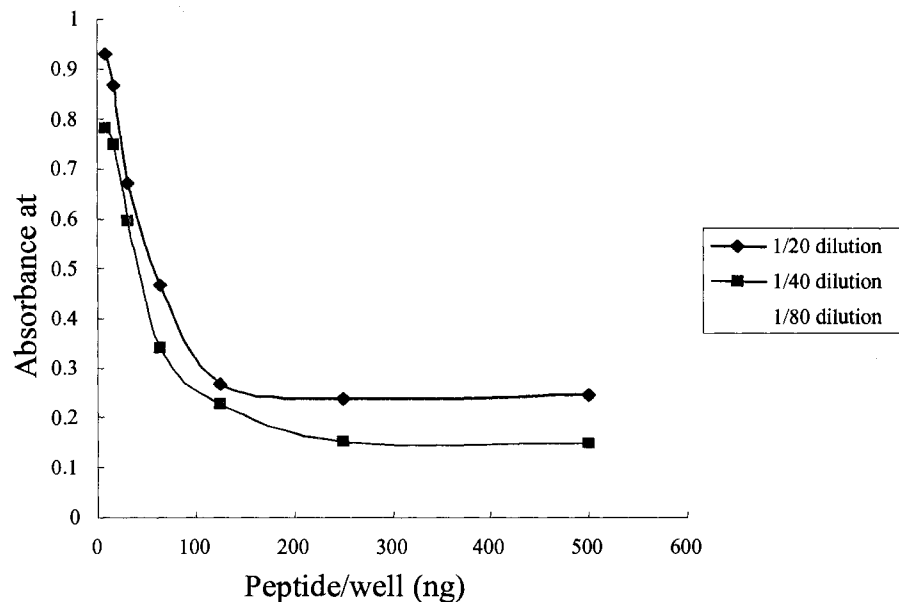


Figure 16. ELISA analysis using different amounts of cBMP4 peptide antigen. Absorbance (405nm) values of samples containing different amounts of synthetic cBMP4 peptide antigen (7.8-500 ng) reacted with three dilutions of pooled immune serum in ELISA using 1:500 dilution of HRP conjugated rabbit anti-mouse secondary antibody.

appropriate conformation without overlapping on each other only when small amount of peptide was used to coat the plates. The highest absorbance detected using 7.8 ng peptide was used to coat the plates.

Immune serum specificity was evaluated by competitive ELISA. Three immune serum dilutions were made and pre-absorbed with 500ng cBMP4 antigenic peptide for an hour. The mice immune response to cBMP4 peptide was specific since immune serum had higher absorbance value than that of pre- absorbed immune serum at all three different dilutions, especially at the highest immune serum dilution (1:80). As we expected, the antibody produced is specific since the higher the antibody dilution, the lower the amount of antibody would present after it was pre-absorbed with the antigen, and therefore, the lower the absorbance (Figure 17).

Monoclonal antibody production. The mouse immune response to the antigenic peptide was weak, and no hybridomas secreting specific antibody were detected (data not shown). Monoclonal antibody production was discontinued.

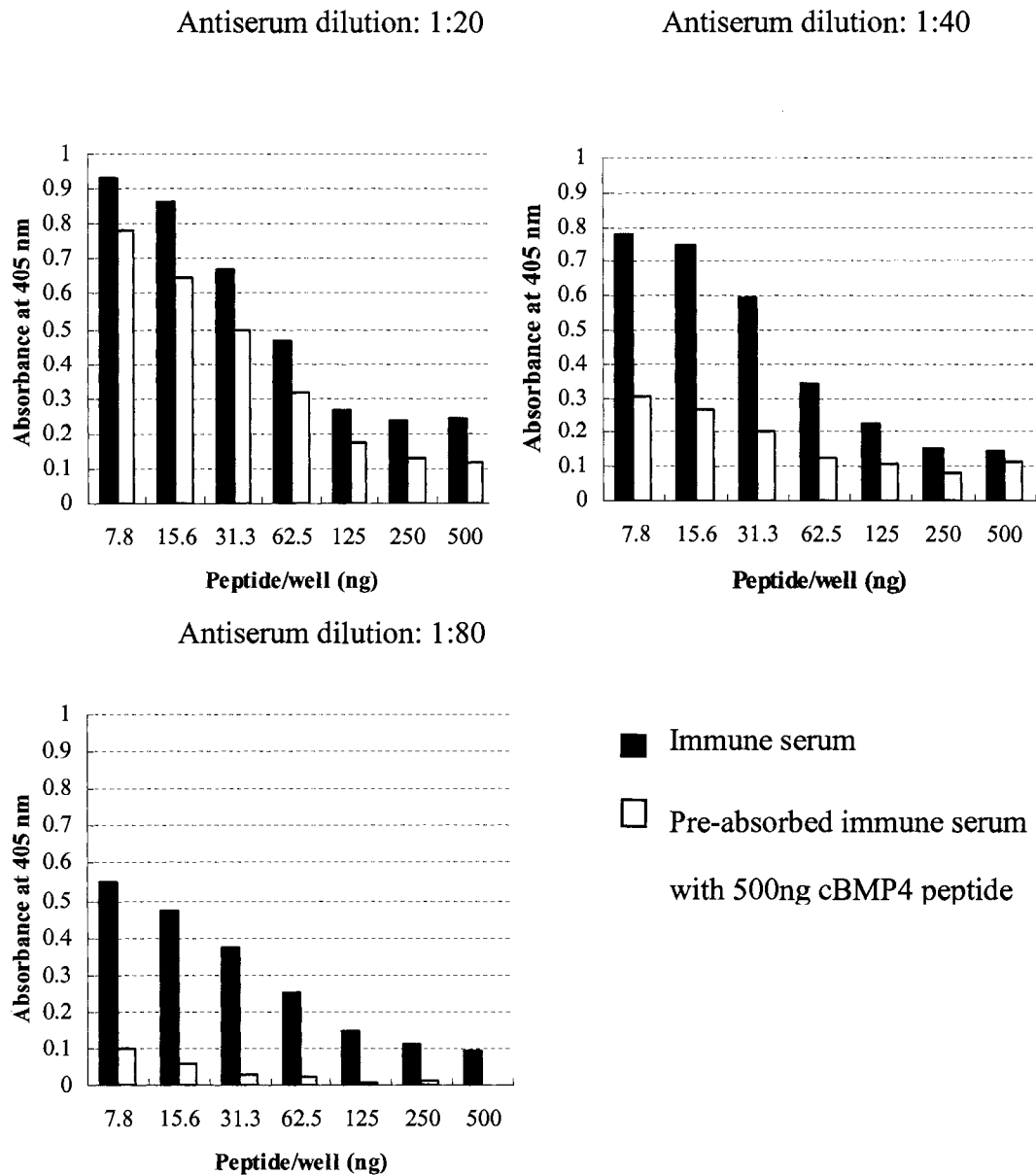


Figure 17. Competitive ELISA analysis. Absorbance (405nm) values of samples containing different amounts of synthetic cBMP4 peptide antigen (7.8-500 ng) reacted with three dilutions of pooled immune serum or pre-absorbed immune serum in competitive ELISA using 1:500 dilution of HRP conjugated rabbit anti-mouse secondary antibody. The pre-absorbed immune serum was prepared by incubation with 500ng cBMP peptide for one hour before adding to the ELISA plates.

Discussion

Cardiac septation defects and valve malformation are the most common congenital birth defects. Recently, congenital heart malformation studies using mouse and chick models have revealed critical yet still poorly understood roles of BMP4 in cardiogenesis. BMP4, for example, appears to be involved both in EMT formation and apoptosis in both endocardial cushions and ventricular myocardium during embryo cardiogenesis (Zhao & Rivkees, 2000). BMP4 has also been identified as a signaling cue from myocardium directly mediating atrioventricular septation (Jiao et al., 2003). Defects in this process cause one of the most common human congenital heart abnormalities, atrioventricular canal defect, but the role of BMP4 in septum formation remains unclear. In order to further explore potential roles of BMP4 during embryonic heart development, time course studies mapping BMP4 mRNA expression patterns have recently been done using *in situ* hybridization (Somi et al., 2004). These expression studies, however, are still incomplete with respect to the stages of development examined and the specific regions of the heart investigated. Moreover, the BMP4 mRNA expression patterns may not necessarily represent BMP 4 protein expression and final deposition patterns. BMP4 is, after all, a secreted protein, and identifying where its origin will not necessarily reveal its field of deposition within the tissue nor its domain of action. Unfortunately, so far only one published paper has focused on immunolocalization of BMP4 protein to examine its expression domain in the development of embryonic endocardial cushions. It is absolutely critical, therefore, to further examine BMP4 protein expression patterns to facilitate our understanding of its function in embryonic heart development.

We selected the chick as research model to carry out a time course study on BMP4 protein expression, although the mouse is often preferred (since it is more closely related to humans than are avians). There are, however, many inherent and practical drawbacks to the use of the mouse as a model organism as opposed to the chick, including much higher animal care/housing expenses, more restrictive regulations regarding the use of mice in animal research, inaccessibility of the embryos during development (and thus an inability to assess the developmental stage of the embryo prior to harvest and an inability to carry out any surgical manipulation, viral transfection or microinjection), the fact that adult animals need to be sacrificed to obtain embryos, the longer gestation time relative to the chick. Like the mouse, the chick too possesses a four chambered heart and a circulatory system extremely similar to that of the human, with a high degree of conservation in structural and regulatory genes. Finally, chick embryos have been extensively studied by embryologists for 100 years, and many developmental stages have a number of well-defined anatomical markers that aid in staging of the embryos.

To localize BMP4 protein expression domains during early embryonic cardiogenesis, a specific, high affinity antibody would prove an invaluable tool, and the homogeneity of antibody isotype, antigenic (epitope) specificity, consistent titer etc exhibited by a monoclonal antibody would be particularly beneficial. Despite the clear utility of such a reagent, however, anti-chick BMP4 antibody is still not commercially available, and the cross-reactivity between anti-human BMP4 mAbs (which are available) against chick BMP4 remains untested by any of the antibody manufactory companies

contacted so far. The short-term goal of this project then was the production of BMP4 protein for use as an immunogen in the generation of a BMP4 mAb. Producing a mAb using a synthetic BMP4 peptide was also sought simultaneously since this approach might succeed in generating a mAb without waiting for large amounts of BMP4 to be purified.

There are three commonly used approaches for monoclonal antibody production based on the different nature of the immunogen: (i) production using natural proteins, (ii) production using recombinant proteins, and (iii) production using one or more synthetic peptides that correspond to specific regions of the native immunogen. Natural proteins are perhaps the ideal immunogens, providing sequence-specific and surface structural epitopes. Natural proteins such as BMP4, however, are often expressed in small amounts. Early attempts to extract BMP protein from bovine bone powder indicated that only 40 µg of protein could be obtained from 40 kg quantities of bovine bone powder (Wozney et al., 1988). Obtaining adequate amounts of BMP4 from chick embryos as immunogen, therefore, is most likely impractical, since it will probably require excessive amounts of embryos as starting material.

Recombinant fusion proteins produced in mammalian cells or bacteria are also used successfully to produce antibodies recognizing specific, three-dimensional determinants. We also adopted this approach, choosing to express a mature cBMP4 fusion protein in both mammalian cells and bacteria cells. Ideally, such an approach would produce enough protein both for use as an immunogen in the production of a mAb and for hybridoma screening purposes.

As a third approach, synthetic peptides conjugated to carrier proteins can sometimes be used to generate antibodies. Although the cost of peptide synthesis is high and the antibody ultimately produced may not recognize the native protein, the advantages of the approach are significant. First, the investigator can, using various bioinformatics tools, design a peptide from a region of the protein that is unique to that protein (especially useful when the protein is one member of a large class of structurally related family members). Secondly, the synthetic peptide can be made easily and quickly, allowing immunization to be started right away. Third, the protein domain recognized by the antibody is unambiguously defined, allowing its use in structural antigen mapping studies. Finally, the defined nature of the immunogen allows the immunogenic peptide to be used later as a blocking peptide, a very valuable reagent when trying to prove specificity of binding. Considering the time frame of this project and the intrinsic difficulty of recombinant protein production, the use of a synthetic peptide was deemed a viable approach for antibody production, and worth an effort despite its disadvantages.

Recombinant Protein Expression in Mammalian Cells

Mammalian cells are commonly used for production of recombinant proteins, especially those of vertebrate origin. In contrast to lower eukaryotes or prokaryotes, mammalian cells possess the appropriate protein processing machinery and often provide active recombinant proteins that possess important post-translational modifications (glycosylation, phosphorylation, proteolytic modification, etc.) required for *in vivo* function. Stable expression technologies, based on chromosome-integrated plasmid sequences and amplifiable expression cassettes such as the dihydrofolate reductase or

glutamine synthetase system, have been shown to provide high levels of recombinant protein expression up to the grams-to-kilograms scale. There are, however, several disadvantages associated with stable expression systems. Development of stable cell lines requires a considerable investment in time, expensive additional reagents (especially selection antibiotics), human resources and lab equipment. In contrast, transient expression provides an economic alternative approach for protein production in the milligram-to-gram scale, which is more suitable for small-scale research purposes.

In order to get proper folding protein with bioactivity, the best approach for recombinant protein production is to produce native cBMP4. Due to intrinsic difficulties in cloning the entire coding region of BMP4, however, the mature region of BMP4 was selected instead. Masuhara et al. (1995) found that homodimeric protein can be used to produce monoclonal antibody that can also recognize the native dimeric BMP protein. In this study, the mature region of chick BMP4 was cloned into the mammalian expression vector pcDNA4 /HisMax, and then transiently expressed in COS-7 cells. The fusion mcBMP4 was apparently produced as a monomeric protein with molecular weight about 16.2 KD as shown by western blotting, suggesting that the disulfide bond was not linked through the seventh cysteine residue intermolecularly to form the BMP4 homodimer (as opposed to native BMP4) although mammalian cells should provide all the machinery necessary for protein dimerization. One possible explanation of this phenomenon is that either the absence of both the signal peptide and the prodomain of the BMP4 or the addition of the N-terminal fusion tag may greatly impact the BMP4 protein processing and trafficking in the mammalian cells, with the result that the protein dimer is not

formed effectively. It is also possible that without the prodomain, the recombinant BMP4 protein is not folded in the correct conformation, in turn causing misalignment of the critical cysteine residues and thereby preventing formation of the homodimer. The fusion protein was folded in such a way that the binding of the purification tag (especially the 6-histidine residue) to the nickel-chelating column was prevented. The fact that mcBMP4 was only present in the crude cell lysates and the nickel affinity column flow-through fraction during protein purification (instead of column elution fractions as expected) tends to support this hypothesis.

Besides the problem associated with protein misfolding, the level of fusion protein expression was low. No strong bands with expected molecular weight of 16.2KD were detected in the SDS-PAGE gels following staining by Coomassie Blue. To further explore the reasons underlying this low level of expression, three key features critical to optimal small scale transient transfection and recombinant protein expression in mammalian cells were evaluated: (i) the nature of the host cells, (ii) the nature of the expression vector, and (iii) the nature of the transfection system.

i) Host Cells. Three cell types have been the most popular for transient recombinant protein expression: human embryonic kidney (HEK)-293 cells, COS cells and baby hamster kidney (BHK) cells. Interestingly, although CHO cells (the most widely used host for stable recombinant protein expression) are more frequently used for recombinant BMP protein expression, they have failed so far to give satisfying expression levels when transfected transiently (Wurm & Bernard, 1999). Like CHO, COS cells are routinely grown as adherent cultures, can be grown to fairly high density,

can be adapted to a variety of growth media etc. Human recombinant BMP4 was first transiently expressed in COS-1 cells to determine the *in vivo* activities of the BMP polypeptides (Wozney et al., 1988). Fifteen years later, another BMP family member structurally similar to BMP4 (human recombinant BMP7) was successfully produced as a mature, disulfide-linked, biologically active homodimer in COS-7 cells (Lee et al., 2003). Those studies supported the idea that at least some clones of COS cells could serve as a functional host for recombinant BMP4 expression, and served as a major justification for our selection of COS-7 cells in this study. The COS-7 cell is an African green monkey kidney fibroblast-like cell line suitable (like most other COS cell lines) for transfection by vectors requiring expression of SV40 T antigen, and will allow episomal amplification of plasmids containing a SV40 origin of replication. The use of such plasmids as expression vectors, therefore, would be expected to increase recombinant protein expression levels by permitting more plasmid copies to persist in the transfected cells throughout the production phase (Van craenenbroeck, Vanhoenacker, & Haegeman, 2000).

ii) Expression vector. There are many expression vectors available commercially. One important issue for high level recombinant protein expression is to use vectors with promoters that are highly active in the host cell line chosen. One example of such a strong, constitutively active promoter active in COS cells is that adapted from the human cytomegalovirus (CMV). Plasmids that carry an SV40 origin of replication will also enable vector replication in COS cells (which constitutively produce the SV40 T antigen required for SV40 genome replication), thereby increasing the plasmid copy number and

thus the transcriptional capacity for recombinant protein production. Another big consideration in vector choice is the presence of structural modification cassettes (which encode protein purification motifs or tags) and the ease with which those modifications to the recombinant protein will facilitate downstream purification. The pcDNA4/HisMax vector from Invitrogen was selected because it carries a CMV promoter, possesses an SV40 origin of replication and produces a recombinant 6-His tagged protein, allowing convenient and efficient metal-chelation affinity purification. It is instructive to note, for example, that the pcDNA3.1/HisMyc vector (which possesses a backbone essentially identical to that of pcDNA4/HisMax) was able to replicate up to 10,000 copies in cells expressing large T antigen (Chittenden, Frey, & Levine, 1991). Besides high copy number, our vector also contains a QBI SP 163 translational enhancer, which increases expression of recombinant protein via a cap-independent translation mechanism. Other useful features of this vector include the presence of the Xpress epitope (which can facilitate protein detection *in vivo*) and the presence of an Enterokinase cleavage site (which allows removal of N- terminal tag from to create the native protein).

iii) Transfection system. The transfection system is another important factor to consider about because it is related to gene transfer efficacy. There are many highly effective gene transfer reagents and methodologies commercially available: (a) the DEAE-dextran method, (b) the calcium-phosphate method, (c) electroporation, (d) ballistic methods, (e) transfection using recombinant viruses, and (f) lipid and liposome-mediated transfection. DEAE-dextran was one of the first chemical reagents used for transfer of nucleic acids into cultured mammalian cells. DEAE-dextran is a cationic

polymer that associates with negatively charged nucleic acid. The positive charged polymer enables the DNA polymer complex to come into closer association with the negatively charged cell membrane, ultimately facilitating uptake into the cells by endocytosis.

Calcium phosphate co-precipitation is also widely used for transfection. Mixing DNA with calcium chloride in PBS will allow the mixture to precipitate and disperse on to the cultured cells. The precipitates can then be taken up by the cells via endocytosis or phagocytosis. This method is widely used because the components are easily available and reasonable in price, but the transfection efficiency is low with low reproducibility.

Electroporation is one of the physical methods for gene transfer. The mechanism for entry into the cell is based upon perturbation of the cell membrane by an electrical pulse, which forms pores that allow the passage of nucleic acids into the cell. Although high transfection efficiency can be achieved using electroporation, the technique requires an expensive electroporator and the cell mortality is high. In addition, fine-tuning and optimization for duration and strength of the pulse for each type of cell are critical to achieve a fine balance between conditions that allow efficient delivery and conditions that kill cells.

Liposome mediated delivery has been widely used to deliver DNA into cells. Unlike the DEAE-dextran or calcium phosphate chemical methods, liposome mediated nucleic acid delivery can be used for *in vivo* transfer of DNA and RNA to animals and humans. Invitrogen's Lipofectamine 2000 is one of many cationic lipid-based transfection reagents presently on the market. Lipofectamine is formulated to give high

transfection efficiency with little toxicity to cells. Invitrogen claims that this reagent gives the highest protein expression levels in the widest variety of adherent and suspension mammalian cell lines compared to similar products. Researchers found that greater than 90% efficiency can be achieved consistently for use lipofectamine to transfect a range of cell lines such as HEK-293, CHO and COS-7. Lipofectamine 2000 was used as the transfection agent in this study and it indeed showed very high transfection efficiency (more than 95%).

In this study, the design of the mammalian expression system was carefully weighed against the basic criteria discussed above. Since the low expression level does not appear to be due to the cell line, expression vector or transfection method, the possibility that low protein expression is largely dependent on the nature of the protein must be considered. In fact, a previous study also showed that the transient expression level of BMP proteins were found very low in COS cells. In that study, the level of expression was dependent on the individual BMP being expressed, but in the best case there was less than 1 percent of the total protein produced (Wozney et al., 1988). Another explanation for the observed low protein expression may be protein misfolding, with the misfolded protein perhaps being targeted for lysosomal degradation.

Recombinant Protein Expression in E. Coli

Relative to mammalian cells, expression in *E. coli* is simple and inexpensive. Because of the difficulties encountered in producing large amounts of mcBMP4 in mammalian cells (and associated protein purification problems discussed in the above section), as an alternative approach, expression of mcBMP4 in a bacterial system (*E. coli*,

BL-21 strain) was attempted to produce sufficient protein for immunization. Monomeric mouse mature BMP4 produced in *E. coli* might elicit the production of a monoclonal antibody that could recognize the native dimeric BMP protein.

E. coli is a gram-negative bacterium containing two membranes: (i) an inner membrane separating the cytoplasm from the periplasm, and (ii) an outer membrane to protect the bacterium from the surrounding environment. The heterologous protein expressed in *E. coli* cells can be directed to the reducing cytoplasm or the comparatively oxidizing periplasm, which facilitates disulfide bond formation. Although for heterogeneous protein composed of several disulfide bonds, periplasm expression is an ideal choice, one inherent drawback of targeting protein secretion to the periplasm is that poor secretion of protein across the inner membrane will result in low protein yield upon purification. As a secondary consequence of impeded protein transport across the plasma membrane, accumulation of the recombinant protein in the periplasm can sometimes produce significant cytotoxicity. For that reason, heterologous protein is often targeted in cytoplasm.

In this study, the recombinant GST-mcBMP4 protein yield was relatively low, possibly due to low protein solubility. Although the production of recombinant fusion proteins in *E. coli* is a well- established technology, some of these factors that may limit production and purification of soluble fusion proteins include: (i) host strains, (ii) the nature of the expression vector, (iii) growth conditions encountered by the recombinant organism, and (iv) the conditions used to induce recombinant protein synthesis.

i) Host strain. A wide variety of *E. coli* host strains can be used for cloning and expressing recombinant protein. Strains deficient in known cytoplasmic protease gene products, such as *Lon*, *OmpT*, *DegP* or *HtpR* may aid in the expression of fusion proteins by minimizing the effects of proteolytic degradation by the host. An example of such a host strain is *E. coli* BL-21, a strain defective in *OmpT* and *Lon* protease production. BL-21 is a well-established strain proved to be able to express the fusion protein in a soluble, intact form. In our project, BL-21 strain was used for cBMP4 protein expression.

ii) Expression vector. A glutathione S-transferase (GST) based gene fusion and expression vector, pGEX-KG, was generously provided by Dr. Julio Soto (whose laboratory routinely uses this vector for recombinant protein expression). This vector is a versatile system for the expression, purification, and detection of fusion proteins produced in *E. coli*, and is based on inducible, high-level expression of a gene (or gene fragment) fused in frame with the *Schistosoma japonicum* GST coding region. Expression in *E. coli* yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. Expression is under the control of the *tac* promoter, which is induced by the lactose analog IPTG. All pGEX vectors are also engineered with an internal *tacIq* gene, the product of which is a repressor protein that binds to the operator region of the *tac* promoter to prevent expression until induction by IPTG, thus maintaining tight control over expression of the insert. In addition to offering chemically inducible, high-level expression, the vectors allow mild elution conditions for release of fusion proteins from the affinity medium. The protein produced would accumulate within the cell's cytoplasm.

iii) Growth conditions encountered by the recombinant organism. Growth parameters are critical in regulating the yield of non-degraded fusion protein. Important variables include growth temperature, duration of the induction period, degree of culture aeration and cell density, the optimization of which often requires striking a balance between yield and quality of the desired recombinant protein. Low temperature growth conditions, for example, can sometimes minimize protein misfolding. This, in turn, will increase protein quality (where properly folded protein is considered to be of higher quality). Since properly folded protein tends to be more soluble, such growth conditions also tend to minimize protein aggregation, increasing potential recovery and yield. Cells grow much slower, however, at reduced temperatures. The decreased cell density tends to reduce the yield. In this experiment, due to time, expense and cell culture system limitations, growth temperatures below 30°C, changes in aeration, nutrient supplementation during induction, etc. were not tested. We found cultured cells at 30°C resulted in no significant improvement of protein quality or yield.

iv) The conditions used to induce recombinant protein synthesis. Many heterogeneous proteins fail to fold into their native state when overexpressed in *E.coli*. Instead, they are either degraded by the cellular proteolytic machinery or accumulated in insoluble form, typically as inclusion bodies. Inclusion bodies are large in size, resist proteolysis and hence can be easy to purify from other cellular components. Purified, insoluble inclusion bodies can be solubilized under strong denaturing conditions such as 8 M urea or 6 M guanidinium chloride, and removal of denaturants sometimes allows refolding of the denatured protein. Unfortunately, the best conditions for refolding are

empirically determined from a set of general guidelines for refolding (Buchner & Rudolph, 1991) and can be painstaking to perform. Optimizing inducing conditions could also potentially increase protein yield through increasing protein solubility. The inducing conditions include varying the concentration of IPTG and duration of the induction time. We observed that varying IPTG induction conditions (0.1mM to 1 mM IPTG) or duration of the induction time made no significant difference in protein solubility or yield.

Monoclonal Antibody Production

We designed and obtained synthetic peptides for use in immunizing mice for antibody production. This approach, however, did not prove successful. The mouse immune response to the peptides was very low, producing antisera with only weak immunoreactivity (maximum immune serum titer reached a value of only 80). Due to the low immune response, the hybridomas we obtained following fusion were incapable of producing antibodies of any significant titer against BMP4. Factors that could contribute to low mouse immune responses included: i) choice of host, ii) peptide selection, iii) selecting the protein carrier and iv) coupling strategy.

i) Choice of host. The animal chosen for immunization should be genetically very different from the animal chosen as the source of the immunogen. In order to achieve a maximum immune response, it is important to avoid self-recognition of the immunogen by the host animal. Although selection of mouse as the host to produce anti-chick BMP4 antibody seems to be a good choice, there is a large homogeneity of BMP4 across the

species. Thus the mouse may have some self-tolerance for this protein, which may contribute to the low immune response.

ii) Peptide selection. There are several elements that may impact the antigenicity of the peptide. First, the selected region should be unique. The region of the peptide selected should be from the mature region, should not possess a site of glycosylation and should represent a unique sequence that would help ensure specificity to the target protein. Second, the peptide should be hydrophilic, flexible and accessible. Since most naturally occurring proteins in aqueous solutions have their hydrophilic residues on the surface and their hydrophobic residues buried in the interior, the selected peptide should be hydrophilic, surface oriented and flexible in order to produce antibody recognize the native protein. The highest point of average hydrophilic for a series of contiguous residues is usually at or near an antigenic determinant (Hopp & Woods, 1981). Because the C-termini or N –termini of proteins are often exposed and have a high degree of flexibility, they are usually a good choice for generating anti-peptide antibodies directed against the intact protein. In our case, the peptide we selected is located in the N-terminal with high accessibility, hydrophilicity and flexibility. Third, the structure of the peptide should be accessible. If the 3D structure of the protein is known, this can greatly assist in choosing exposed epitopes. Surface regions or regions of high accessibility often border helical or extended secondary structure regions. In addition, sequence regions with beta-turns or amphipthic helix character have been found to be antigenic (Parker & Hodges, 1991). Fourth, the length of the peptide should be appropriate. There are two different thoughts on the topic of peptide length. One suggests that long peptides

(20-40 amino acids in length) are optimal because it increases the number of possible epitopes. The other suggests that smaller peptides are sufficient, and their use ensures that the site-specific character of anti-peptide antibodies is retained. Any peptide selected must be chemically synthesizable and should be soluble in aqueous buffer for conjugation to carrier proteins. Peptides longer than 20 residues in length are often more difficult to synthesize with high purity because there is greater potential for side reactions, and they are likely to contain deletion sequences. On the other hand, short peptides (<10 amino acids) may generate antibodies that cannot recognize the native protein. The typical length for generating anti-peptide antibodies is in the range of 10-20 residues. Peptide sequences of this length minimize synthesis problems, are reasonably soluble in aqueous solution and may have some degree of secondary structure. The peptide length we selected is within this range.

iii) Selecting the protein carrier. Conjugation to a carrier protein is important because peptides are small molecules and do not tend to be immunogenic. The carrier protein contains many epitopes that stimulate T helper cells, which helps induce the B cell response. Many different carrier proteins can be used for coupling to synthetic peptides. The most commonly selected carriers are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). The higher immunogenicity of KLH often makes it the preferred choice. Another advantage of choosing KLH over BSA is that BSA is used as a blocking agent in many experimental assays. KLH was used for conjugation in our project.

iv) Coupling strategy. The coupling site and technique utilized also play important roles in determining the reactivity of the antibodies. Generally, peptides can be coupled to other proteins by utilizing: a) A free NH_2 or COOH (recommended for peptide when Cysteine cannot be added to the peptides); b) A free Cysteine group (available as part of the sequence or added at the N or C-terminus). The coupling method utilized in our project was Glutaraldehyde method, which is empirically selected based on the recommendation of the peptide synthesis scientist at ADI International.

Glutaraldehyde is a bifunctional coupling reagent that links two compounds through their amino groups. Glutaraldehyde provides a highly flexible spacer between the peptide and carrier protein for favorable presentation to the immune system. Unfortunately, glutaraldehyde is a very reactive compound and will react with Cysteine, Tyrosine and Histidine to a limited extent. The result can be a poorly defined conjugate. The glutaraldehyde method is particularly useful when a peptide contains only a single free amino group at its amino terminus. If the peptide contains more than one free amino group, large multimeric complexes can be formed, which are not well defined, but are highly immunogenic.

Although the selected cBMP4 peptide meets all these criteria, immunogenic capacity of a peptide not only depends on intrinsic chemical properties of the peptide but also on extrinsic factors such as the host immunoglobulin repertoire, self-tolerance and various cellular and regulatory mechanisms definable only in the context of the host (Van Regenmortel, 2001). A strong humoral immune response directed against the peptide is thought to require the stimulation of two cell types. Specific pre-B lymphocytes must be

stimulated by the binding of the peptides to their immunoglobulin receptors. These cells mature into the antibody secreting lymphocytes. A strong response also requires the stimulation of helper T lymphocytes by the interaction of the carrier portion of the peptide-carrier complex with the appropriate T lymphocyte cell surface receptor. Thus, an effective immune response will largely depend on the interaction of peptide carrier with both B cells and T cells. The immunogenicity of a peptide is not completely understood, but low mice immune response may be influenced by several uncontrollable factors in the host such as accessibility to binding by the B cell surface receptors, ability of the carrier to stimulate helper T cell production, stability of the immunogen with respect to macrophage degradation and presentation to the immune system, size of the reacting B and T cell populations, etc. (Shinnick, Sutcliffe, Green, & Lerner, 1983).

Future Directions

This study has shown that recombinant mature chick BMP4 (mcBMP4) can be expressed in both COS-7 cells and *E. coli* cells, but at levels too low to be of any practical use for the production of immunogenic protein and monoclonal antibody generation. It was unsuccessful to express mature BMP4 protein as a monomer. Producing correctly folded mcBMP4 protein may require cloning and expressing of a full length BMP4 pre-protein. In that case, firstly, it would be necessary to redesign both the PCR primers (to include restriction enzyme sites for oriented cloning) and the expression vector (to allow production of a fusion protein carrying a C-terminal tag immediately downstream of an enterokinase site). The vector modifications would hopefully allow proper folding of the BMP4 precursor and subsequent proteolytic processing of the pre-

protein into a mature disulfide-linked BMP4 dimer. Ideally, such folding and proteolytic processing would occur normally for the recombinant BMP4 fusion protein despite the presence of the C-terminal purification tag (His domain, GST domain, etc.). If it was later decided to remove the tag, the presence of the enterokinase cleavage site would facilitate this effort. The stop codon at the end of the chick BMP4 DNA coding sequence, for example, would need to be altered (using site directed mutagenesis) in order to allow expression of cBMP4 with fusion tag at the end.

Secondly, given the known processing requirements and their apparent necessity for protein stability, expression in eukaryotic cells will be required. Switching the position of the tag to the C-terminal end would also facilitate inclusion of the secretion signal at the N-terminus. In order to improve the protein yield, generation of a stable expression cell line might be a reasonable alternative to transient expression, especially considering the low expression level observed with the current construct. Finally, the cell line selected for stable expression could be adapted for suspension culture since the culture volume can be easily expanded prior to use in protein purification. This would facilitate starting from a greater initial number of cells and aid in the isolation of more BMP4 protein.

The use of synthetic peptides as immunogens for monoclonal antibody production should be pursued using a combination of two or more peptides together to immunize mice, stimulating a stronger immune response than a single peptide. At least one peptide should be selected from the unique region of the cBMP4 protein, and the others could be selected from evolutionary conserved regions of the cBMP4. Any hybridomas resulting

from this immunization strategy should be screened against the unique region antigenic peptide to minimize the possibility of selecting mAbs that recognize the conserved region. Efforts to design two or three such immunogenic peptides are currently underway.

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Appendix 1. IACUC approval of using mice for monoclonal antibody production.

**San Jose State University
Institutional Animal Care and Use Committee**

LETTER OF OFFICIAL PROTOCOL REVIEW

Date: November 17, 2006

Dear Dr. White

The animal care and use portion of your research proposal indicated below was reviewed by the Institutional Animal Care and Use Committee (IACUC). The status of your proposal is as follows:

Principal Investigator(s): Steven White, Sherry Li

Protocol #: 867

Title: Production of Monoclonal Antibody to Detect Bone Morphogenetic Protein (BMP4) Expression Domain During Chick Heart Development.


The application was approved without modification by the IACUC

Approval date: June 1, 2005 * Expiration Date: May 31, 2007

The IACUC must be informed in writing of any proposed changes to the approved protocol outline and approval must be granted in writing by the IACUC before any change is instituted. If you wish to continue the approved outline beyond the expiration date listed, it is required that you submit a request for protocol approval extension for IACUC consideration in April 2007.

The protocol number (#867) may only be used by the principal investigator and participants included on the approved application form. The protocol number will be required to order animals (maximum of 9 mice and 24 fertile chicken eggs) and be included on grant or contract proposals to fund the project. Please notify the UAC office for placing animal orders or to request the transfer of live animals to or from this approved protocol.

If you have any questions, feel free to contact me at 408-924-4929.


Larry Young, RWT, LATg
IACUC Coordinator

This protocol has been approved as a Health Risk Category One level project (RC-1).

Please refer to the attached risk category description page for relevant personnel safety information pertaining to this study.

Query	287	AGCCCCAAGCACCACGGTTCCCGCAAGAACaaaaaaaaaCTGCCGCCGCCACGCTCTCTAT	346
Sbjct	874	AGCCCCAAGCACCACGGTTCCCGCAAGAACAAAAAAAAACTGCCGCCGCCACGCTCTCTAT	933
Query	347	GTGGATTTTCAGCGACGTGGGTTGGAACGATTGGATCGTGGccccccGGGCTACCAGGCG	406
Sbjct	934	GTGGATTTTCAGCGACGTGGGTTGGAACGATTGGATCGTGGCCCCCCCCGGGCTACCAGGCG	993
Query	407	TTTTACTGCCACGGGGACTGCCCCCTTCCCTCTGGCCGACCACCTCAACTCCACCAACCAC	466
Sbjct	994	TTTTACTGCCACGGGGACTGCCCCCTTCCCTCTGGCCGACCACCTCAACTCCACCAACCAC	1053
Query	467	GCCATCGTGCAGACGTTGGTCAACTCCGTCAACTCCAGCATCCCCAAGGCCTGCTGCGTG	526
Sbjct	1054	GCCATCGTGCAGACGTTGGTCAACTCCGTCAACTCCAGCATCCCCAAGGCCTGCTGCGTG	1113
Query	527	CCCACGGAGCTGAGCGCCATCTCCATGCTCTACCTGGATGAGTATGACAAGGTGGTGCTG	586
Sbjct	1114	CCCACGGAGCTGAGCGCCATCTCCATGCTCTACCTGGATGAGTATGACAAGGTGGTGCTG	1173
Query	587	AAAAACTACCAGGAGATGGTGGTGGAGGGGTGCGGGTGCCGCTGACCCCTTCCC	640
Sbjct	1174	AAAAACTACCAGGAGATGGTGGTGGAGGGGTGCGGGTGCCGCTGACCCCTTCCC	1227

File: C2_T7.ab1 Run Ended: May 19, 2006, 4:39:16 Signal G:197 A:189 T:194 C:229 Comment: bands
Sample: C2-T7 Lane: 46 Base spacing:15.24 1264 bases in 16301 scans



Appendix 4. Verification of mcBMP4 insert identify and orientation in PGEX-KG *E. coli* expression vector by BLAST search.

Gallus gallus bone morphogenetic protein 4 (BMP4), mRNA

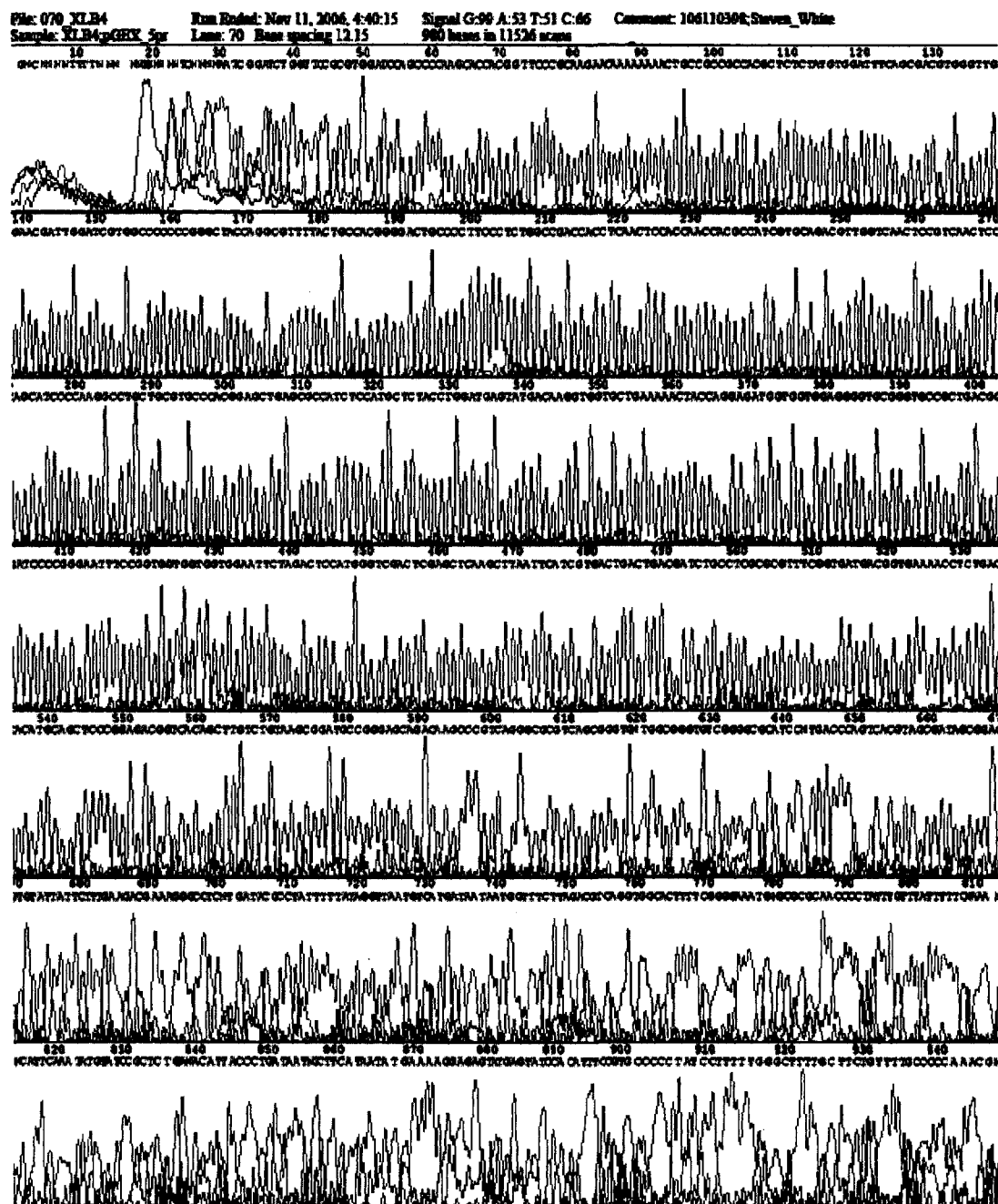
Score = 647 bits (347), Expect = 0.0

Identities = 347/347 (100%), Gaps = 0/347 (0%)

Strand=Plus/Plus

Query	54	CAGCCCCAAGCACCACGGTTC	CGCAAGAAC	aaaaaaaaa	CTGCCGCGCCACGCTCTCTA	113
Sbjct	873	CAGCCCCAAGCACCACGGTTC	CGCAAGAAC	AAAAAAAAA	CTGCCGCGCCACGCTCTCTA	932
Query	114	TGTGGATTTCAGCGACGTGGG	TTGGAACGATTGGATCGTGG	ccccccc	GGGCTACCAGGC	173
Sbjct	933	TGTGGATTTCAGCGACGTGGG	TTGGAACGATTGGATCGTGG	CCCCCCC	GGGCTACCAGGC	992
Query	174	GTTTTACTGCCACGGGGACTG	CCCCCTTCCCTCTGGCCGACC	ACCTCAACTCCACCAACCA		233
Sbjct	993	GTTTTACTGCCACGGGGACTG	CCCCCTTCCCTCTGGCCGACC	ACCTCAACTCCACCAACCA		1052
Query	234	CGCCATCGTGCAGACGTTGGT	CAACTCCGTCAACTCCAGCAT	CCCCAAGGCCTGCTGCGT		293
Sbjct	1053	CGCCATCGTGCAGACGTTGGT	CAACTCCGTCAACTCCAGCAT	CCCCAAGGCCTGCTGCGT		1112
Query	294	GCCCACGGAGCTGAGCGCCAT	CTCCATGCTCTACCTGGATGAG	TATGACAAGGTGGTGCT		353
Sbjct	1113	GCCCACGGAGCTGAGCGCCAT	CTCCATGCTCTACCTGGATGAG	TATGACAAGGTGGTGCT		1172
Query	354	GAAAAACTACCAGGAGATGGT	GGTGGAGGGGTGCGGGTGCCG	CTGAC		400
Sbjct	1173	GAAAAACTACCAGGAGATGGT	GGTGGAGGGGTGCGGGTGCCG	CTGAC		1219

Appendix 5. Verification of mcBMP4 insert identify and orientation in PGEX-KG *E. coli.* expression vector by DNA sequencing.



Appendix 6. Chick BMP4 DNA sequence (Open Reading Frame) and translated chick BMP4 protein sequence. cBMP4 DNA sequence is showed in black while cBMP4 protein sequence is showed in blue. Mature region of cBMP4 protein is highlighted in yellow. The potential glycosylation sites are marked in boxed letters.

```

1      M I P G N R M L M V I L L C Q V L L G G
   atgattcctggtaaccgaatgctgatgggtcatcctactatgccaaagtcctgctgggaggt 60
61     T N H A S L I P E T G R K K V A E L Q G
   accaaccatgctagcctgatccccgagaccggcaggaagaaagtcgcagagctcagggga 120
   Q A G S G R R S A Q S H E L L R G F E T
121    caagccggatccggacgccgtctgcccgaagccatgaactcttgcggtttcgaaacg 180
   T L L Q M F G L R R P Q P S K S A V I
181    actctgctgcagatgtttgggtgcgaaggcggccccagcccagcaaatcagccgtcatc 240
   P S Y M L D L Y R L Q S G E E E E R S L
241    ccagttacatgctggatctctaccggctccagtcgggagaagaggaggagaggagcctc 300
   Q E I S L Q Y P E R S A S R A N T V R S
301    caggagatcagcctgcagtaccggagcgatctgccagccggccaacaccgtgaggagc 360
   F H H E E H L E S V P G P S E A P R I R
361    tccaccatgaagagcacctggagagcgtgccgggtcccagcgaagcgccgcgatccgc 420
   F V F N L S S V P D N E V I S S E E L R
421    ttcgtcttcaacctcagcagcgtgccggacaacgaggtgatctcgtcggaggagctgcgg 480
   L Y R E Q V E E P S A A W E R G F H R I
481    ctgtaccgggagcaggtggaggagccgagcgcggcgtgggagaggggttccaccggata 540
   N I Y E V M K P L S E R S Q A I T R L L
541    aacatttacgaagtgatgaagccgctgtcggagcgtcgcaggccattacgcgcctgttg 600
   D T R L V H H N V T R W E T F D V S P A
601    gacacgcggttggtgcaccacaacgtgacgcgctgggagacctttgatgtgagcccagcc 660
   V I R W T K D K Q P N H G L V I E V T H
661    gtgatccgggtggaccaaggacaagcagcccaaccacgggctggtgatcgaggtgaccac 720
   L H Q A Q T H Q G K H V R I S R S L P Q
721    ctccaccaggcacagactcatcagggcaaacacgtcaggattagccgatctttacctcag 780
   G H G G D W A Q L R P L L V T F G H D G
781    gggcacggcggggactgggcgcagctcaggccgctcctggtcaccttcgggcacgacggg 840
   R G H A L T R R A R R S P K H H G S R K
841    cgaggccacgcgctgaccgcagggcccgccgagccccaagcaccacgggttcccgaag 900
   N K K N C R R H A L Y V D F S D V G W N
901    aacaaaaaaaaactgccgccccaacgctctctatgtggatttcagcgacgtgggttggaa 960
   D W I V A P P G Y Q A F Y C H G D C P F
961    gattggatcgtggcccccccggtaccaggcggtttactgccacggggactgcccttc 1020
   P L A D H L N S T N H A I V Q T L V N S
1021   cctctggccgaccacctcaactccaccaaccacgccatcgtgcagacgttggtcaactcc 1080
   V N S S I P K A C C V P T E L S A I S M
1081   gtcaactccagcatccccaaggcctgctgcgtgccacggagctgagcgccatctccatg 1140
   L Y L D E Y D K V V L K N Y Q E M V V E
1141   ctctacctggatgagtatgacaagggtggtgctgaaaaactaccaggagatggtggtggag 1200
   G C G C R *
1201   ggggtgcgggtgccgctga

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Appendix 7. List of commonly used abbreviations in this study.

Abbreviation	Full Term
AVCD	atrioventricular canal defect
ASP	atrial septum primum
OT	outflow tract
AVC	atrioventricular canal
EMT	epithelial-mesenchymal transformation
TGF- β	transforming growth factor- β
BMP	bone morphogenetic proteins
PBSM	parabronchial smooth muscle
IPTG	isopropyl- β -D –thiogalactoside
YTA	Trytone yeast ampicillin
GST	1-chloro-2,4-dinitrobenzene
CDNB	1-chloro-2,4-dinitrobenzene
GST	glutathione S-transferase
KLH	keyhole limpet hemocyanin
GLUT	glutaraldehyde
PMSF	phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride)