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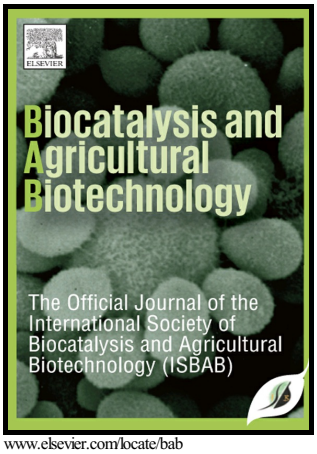

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Cloning and expression of Human Bone Morphogenetic Protein-2 gene in *Leishmania tarentolae*

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Abstract

Several expression hosts have been employed for the production of bone morphogenetic protein-2 (BMP-2), inducing de novo bone formation and is widely used in clinical applications. However, every expression system has its merits and demerits. For example, although *E. coli* is simple and robust, but inept to make disulfide bond; hence, heterologous proteins are produced in the form of inclusion bodies, entailing *in vitro* refolding strategies. On the other hand yeast, which is a lower eukaryote, is able to perform glycosylation of the recombinant proteins. However, its glycosylation pattern is different from human beings. Ultimately, *Leishmania tarentolae*, a parasite protozoan of the lizard, unifying both the characteristics of prokaryotes and eukaryotes, has been introduced for the expression of glycoproteins. It is capable of making disulfide bonds, expressing correctly folded and biologically active recombinant proteins. In the current study, we successfully cloned and expressed proBMP-2 gene in *L. tarentolae*. Integration of the gene was confirmed by PCR; whereas, transcription of the coding gene was verified by back translating the target mRNA. Besides, the expression of BMP-2 was assessed by SDS-PAGE and Western blotting. A 12kDa band was obtained, equivalent to monomeric form of BMP-2.

Keywords: Bone morphogenetic protein 2, Morphogenesis, Growth differentiation factor 2, Osteogenesis, Spinal cord injuries, Glycosylation

1. Introduction

Bone remodeling is a natural phenomenon in which bone formation and bone resorption occur spontaneously, due to the intracellular communication of osteoblasts osteoclasts. Osteoblast cells induce the formation of bones while osteoclasts are bone resorbing cells. Bone remodeling completes in three consecutive steps. Firstly, osteoclasts digest the old bone; secondly, mononuclear cells appear on the surface of bone, and finally bone formation (Hadjidakis and Androulakis, 2006). A number of factors; such as, transforming growth factor-β (TGF-β), insulin like growth factor (IGF-1), interleukine-6 (IL-6), and bone morphogenetic proteins (BMPs) are involved in bone formation. However, only BMPs have the potentiality to induce de novo bone formation (Garimella et al., 2008). The seminal work of Urist (Urist, 1965) has unveiled the notion that bone morphogenetic proteins (BMPs) are the key players in bone regeneration.

Actually BMPs are multifunction cytokines, belonging to the TGF-β superfamily. Studies conducted on rodents have revealed that BMPs induced ectopic bone formation when placed under the skin of animals. It has also been demonstrated that BMPs regulate the process of embryogenesis and control the differentiation of bones, tissue, tendons, and ligaments. Due to their critical role in bone regeneration, scientists have started to study the mechanism of action of BMPs in various organs. The signaling mechanism of BMPs has been reviewed in detail (Oshin and Stewart, 2007). Some of the well-known BMPS including, BMP-2, BMP-4, BMP-6, and BMP-7 have osteoinductive properties. BMP-2 and BMP-7 have currently been approved by FDA for repairing bone fractures and spine surgery (Kamiya, 2012). Other clinical data demonstrated that BMP-2 successfully induced bone formation in osteoporotic rats (Tang et al., 2008).

Mature BMP-2 is a homodimer, each monomer contains a cysteine knot which is composed of six cysteine residues forming three intrachain disulfide bridges. The formation of hydrophobic core between monomers of BMP-2 structure makes it more stable (Scheufler et al., 1999). In the last decades, BMPs were mostly isolated and purified from bones; unfortunately, the procedure was laborious and time consuming. Repeated extraction increased the yield but reduced the biological activity of BMPs both *in vivo* and *in vitro* (Hu et al., 2004). Various expression systems have been utilized for producing rhBMPs; for instance, it is possible to express rhBMP-2 in Chinese hamster ovary (CHO) cells (Israel et al., 1996, Israel et al., 1992, Wang et al., 1990). Attempts have also been made to express biologically active rhBMP-2 in *E. coli* through *in vitro* refolding of inclusion bodies (Vallejo et al., 2002, Long et al., 2006). Other studies used yeast and transgenic plants for the production of heterologous BMPs in large scale. Recently *L. tarentolae*, a trypanosomatid protozoan parasite of gecko has been utilized for the expression of heterologous proteins. This group of protozoa is rich in glycoproteins and its glycosylation pattern is similar to human mammals (Basile and Peticca, 2009). In current study we successfully cloned and expressed proBMP-2 gene, isolated from mammalian cell line i.e. MG-63, in *L. tarentolae.*

2. Materials and Methods

2.1. Cloning BMP-2 coding cDNA

Osteosarcoma cell line, MG-63 was cultured in DMEM medium (Gibco, USA), supplemented with 10% FBS (Gibco, USA). Messenger RNA was extracted from MG-63 cell line, according to

the manufacturer protocol, using GF-TR-025 RNA isolation kit (Vivantis, Germany). Concentration and purity of mRNA was determined at A260:280 and loaded on 1% agarose gel for visualization. Then 10 µg mRNA having OD >1.7 at A260/280, was reverse transcribed with 2-steps RT-PCR kit (Vivantis, Germany). From cDNA proBMP-2 encoding sequence was amplified using proBMP-2 specific forward and reverse primers, containing SalI and KpnI restriction sites (Table 1). PCR product of proBMP-2 and pTZ57R, a cloning vector both were digested with SalI and KpnI restriction enzyme (Vivantis, Germany).

2.2. Integration of proBMP-2 fragment in PTZ57R

Digests were loaded on 1% agarose gel and after electrophoresis; bands were purified using GF-1 nucleic acid recovery kit (Vivantis, Germany). Purified proBMP-2 gene fragment was then ligated in already digested pTZ57R vector, using T4 Ligase (Fermentas, Lithuania). For the confirmation of ligation, *E. coli* TOP10 strain was transformed with pTZ57R-proBMP-2 cloning vector. Transformed colonies were screened and selected on the base of blue/white colonies on LB agar plates supplemented with ampicillin and X-gal. Then a single white colony was cultured in LB broth, supplemented with ampicillin, cultured overnight and then subjected to plasmid extraction. Purified plasmid was confirmed on 1% agarose gel. Besides, ligation of proBMP-2 gene in cloning vector was also confirmed through PCR, using universal pTZ57R and proBMP-2 specific primers (Table 1).

Table 1. List of primer sequences used in this study

Primers	**Sequence (5' to 3')**
BMP-2 Forward	ATAAGCGACGCAGTTCCGGAGCTGG
BMP-2 Reverse	ATAAGGTACCGCGACACCCACAACC
ptzSeq F	AGTTGGGTAACGCCAGGG
ptzSeq R	TTTCACACAGGAAACAGC
P1442	CCGACTGCAACAAGGTGTAG
A264	CATCTATAGAGAAGTACACGTAAAAG
F 3001	GATCTGGTTGATTCTGCCAGTAG

2.3. Construction of expression vector

After confirmation, our next target was to subclone proBMP-2 gene in pLEXSY-hyg2 expression vector (Jena Bioscience, Germany). Both pTZ57R and pLEXSY-hyg2 expression vector were double digested with SalI and KpnI restriction enzymes (Vivantis, Germany). ProBMP-2 gene fragment and cut pLEXSY, both were purified from gel using GF-1 nucleic acid

recovery kit (Vivantis, Germany). Purified proBMP-2 gene was then ligated in the multiple cloning sites of purified pLEXSY-hyg2 expression vector using T4 DNA ligase. For confirmation of proper ligation, *E. coli* XL1 strain (Stratagene), was transformed with expression vector. From transformed colonies plasmid was purified, using plasmid MiniPrep Kit (Bioneer, Korea), and subjected to double digestion with SalI and KpnI restriction enzymes (Vivantis, Germany). Purified plasmid was further verified through PCR, using pLEXSY specific P1442 and A264 primers (Jena Bioscience, Germany), and proBMP-2 forward and reverse primers (Table. 1). The gene was sequenced and checked for mutation by comparing with the reference sequence in gene bank (NC_000020.11).

2.4. Cultivation of *L. tarentolae*

L. tarentolae strain P10 LEXYcon2 Expression Kit (Jena Bioscience, Germany), was cultured in brain heart infusion (BHI) broth (Heimedia, India), supplemented with hemin (5 µg/ml), penicillin (50 U/ml), and streptomycin (50 µg/ml). Static cultures were cultivated in 25 ml tissue culture flasks, containing 10 ml BHI medium, and incubated at 26^0C in the dark. The medium was diluted at 1:10 into fresh medium twice a week and the number of live cells were counted on hemocytometer.

2.5. Transfection of *L. tarentolae* cells

The pLEXSY-proBMP2 expression vector was purified with plasmid MiniPrep Kit (Bioneer, Korea) and digested with SwaI restriction enzyme. The linearized DNA fragment and cut pLEXSY both were purified from gel using GF-1 nucleic acid recovery kit (Vivantis, Germany). For electroporation about 1×10^8 cells of *L. tarentolae,* cultured for 48 hours, were centrifuged and resuspended in 1ml pre-chilled electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, and 5 mM $MgCl_2$, pH 7.6). Approximately, 5 µg of linearized DNA was used for transfecting 500 µl of *L. tarentolae* suspension in 4mm cuvettes while in other tube cells were treated with empty self-ligated vector. Electroporation was carried out using Gene PulserXcell (Bio-Rad), by giving two pulses of 450 V and 450 µF for 3 millisecond pulse time. Electrophoresed cells were kept on ice for 10 minutes and then moved to BHI-hemin medium, supplemented with 100 µg/ml hygromycin. Finally, treated cells were incubated at 26^0C in the dark for one week. Transformed cells survived in the form of dense cultures on selection medium, while no growth was observed in negative control cells,

transfected with empty vectors. Hygromycin resistant transformed cells were selected and used for further analysis.

2.6. Screening of transformed *L. tarentolae* cells

At molecular level homologous recombination was confirmed by extracting DNA from transformed *L. tarentolae* cells. Then DNA was PCR amplified with F3001 forward primer compatible with *ssu* locus of *L. tarentolae* genome, provided by LEXSYcon2 Expression Kit, (Jena Bioscience, Germany), and proBMP-2 specific reverse primers (Table. 1). While for the analysis of transcription, mRNA was extracted from transformed *L. tarentolae* cells using GF-TR-025 (Vivantis, Germany). And cDNA was synthesized by using 2-steps RT-PCR kit (Vivantis, Germany). PCR product was visualized on 1% agarose gel using ethidium bromide stain.

2.7. Expression analysis through SDS-PAGE and Western blotting

Expression of proBMP-2 gene was confirmed through sodium dodecyl sulfate polyacrylamide (SDS-PAGE) and Western blotting. For this purpose, *L. tarentolae* cells cultured in BHI medium were centrifuged, 20 ml of supernatant was taken and 2 ml of 50% ice cold trichloroacetic acid was added to it, and incubated for 30 minutes on ice. After incubation the solution was centrifuged at 12000 rpm for 5 minutes at 4^0C. The pellet was resuspended in 1 ml acetone and centrifuged again at the same condition. Cell lysate was also evaluated for protein expression. The extracted proteins were analyzed by loading on 12% SDS-PAGE, under non-reducing conditions, stained with Coomassie blue and then destained with destaining solution.

After SDS-PAGE separation, bands from gel were transferred to nitrocellulose membrane, and subjected to Western blotting to confirm the expression of rhBMP-2. The membrane was blocked in buffer containing 3% skimmed milk and then soaked for 2 hours in monoclonal anti-BMP-2 antibody (Abcam, UK). Finally, secondary antibody, conjugated with horseradish peroxidase (HRP), diluted 1:5000 in phosphate buffer saline pH 7.2, containing 0.1% Tween20, was employed for visualization. After 3 times washing, HRP substrate (DAB and H_2O_2) was added and color development was observed (Figure 4).

3. Results:

3.1. cDNA synthesis and cloning vector construction

Extracted mRNA from MG-63 cells was reverse translated to cDNA. proBMP-2 gene sequence was obtained and integrated in the pTZ57R cloning vector. Constructed pTZ57R-proBMP-2 vector was amplified by transforming *E. coli* TOP10 cells. For further confirmation of the construct, plasmid was purified from transformed cells and amplified through PCR with two sets of primers along with negative controls. After loading and gel electrophoresis, a 1300bp band of pTZ57R and 1100bp band of proBMP-2 were visualized (Figure 1 A&B).

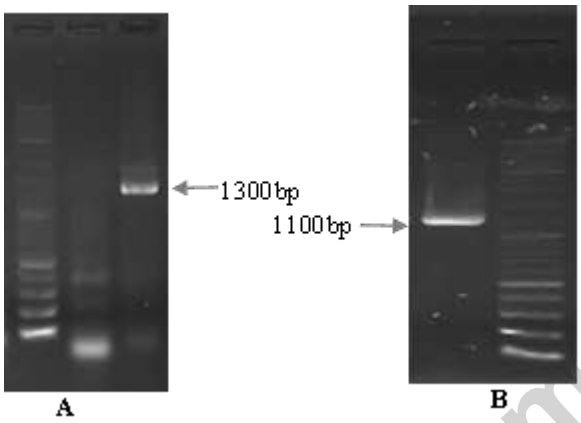

Figure 1 A. Universal PCR product of pTZ57R free of target gene displayed a 1300bp band **B.** BMP-2 specific PCR product of plasmid extracted from white colonies displayed 1100bp band

3.2. Construction of expression vector

pLEXSY expression vector contains *ssu* sites for homologous recombination to facilitate the process of integration in *L. tarentolae* genome. proBMP-2 gene fragment after purification from pTZ57R cloning vector was then introduced to the pLEXSY expression vector and the construct was amplified by transforming *E. coli* XL1 cells. From transformed colonies expression vector was purified and confirmed through PCR, carried out with proBMP-2 and pLEXSY specific primers. On agarose gel a single band of 1100bp was the PCR product of pLEXSY sequence (Figure 1A) and 1100bp was the PCR product of proBMP-2 (Figure 1B).

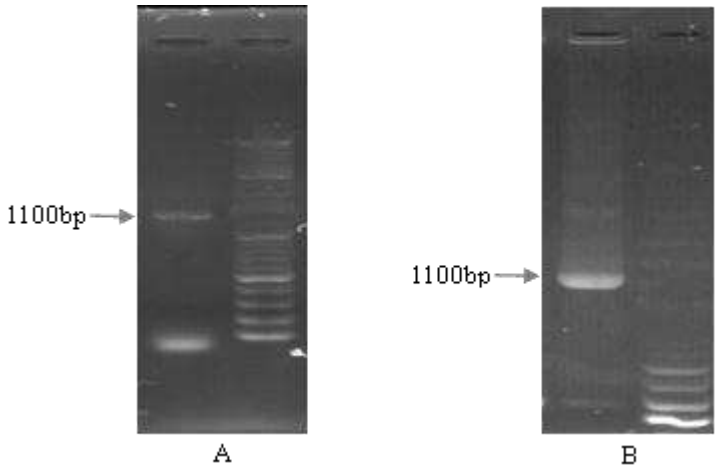

Figure 2. A. Universal PCR product for pLEXSY-BMP-2 construct extracted from E. coli XL1 colonies to confirm ligation of BMP-2 gene in multiple cloning sites. **B.** BMP-2 specific PCR product for pLEXSY-BMP-2 construct extracted from E. coli XL1 colonies to confirm ligation of BMP-2 gene in full length.

3.3. Selection and culturing of transfected *L. tarentolae* cells

Transfected *L. tarentolae* cells survived in the selection medium while those transfected with empty vectors died. Homologous recombination of the linearized pro-BMP-2 gene fragment in the chromosome of *L. tarentolae* was further assessed through PCR. PCR results on 1% agarose gel revealed a 2200bp band of amplified *ssu* locus coupled with proBMP-2 gene. Thus, confirming the chromosomal integration of gene fragment (Figure 3). Furthermore, transcription of the integrated gene was evaluated by reverse transcribing the coded mRNA. The RT-PCR product showed 1100bp band; thereby, confirmed the transcription of integrated gene (Figure 3).

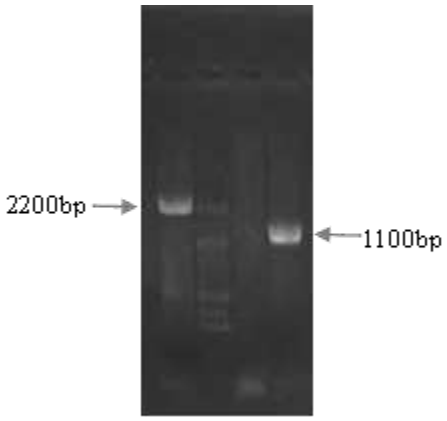

Figure 3 Lane 1; 2200bp band of diagnostic PCR of ssu locus with pBMP-2 gene, **Lane 2;** Ladder, **Lane 3;** Negative control **Lane 4;** 1100bp band of proBMP-2 specific PCR carried out on DNA extracted from Leishmania.

3.4. SDS-PAGE and Western blot analysis

The expression of BMP-2 was checked by isolating the proteins from cell lysate. Proteins loaded on SDS-PAGE revealed a single band. After running SDS-PAGE, bands were transferred to a nitrocellulose membrane and Western blot analysis was carried out by using anti-BMP-2 antibody. A 12kDa band was observed on nitrocellulose membrane (Figure 4), which is approximately equal to the monomeric form of BMP-2. We did not observed the dimeric form of BMP-2.

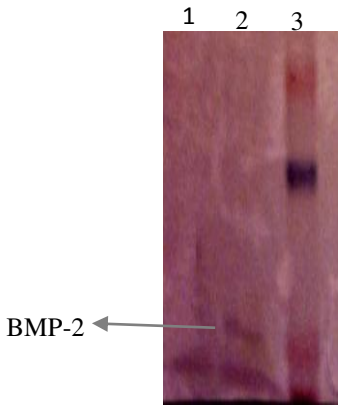

Figure 4 Western blot analysis of expressed protein. Cell lysate was evaluated for protein expression, **Lane1;** Negative control, **Lane 2;** Recombinant BMP-2, and **Lane 3;** Pre-stained ladder.

4. Discussion

BMP-2 is a homodimer, linked through a disulfide bridge and expressed as propeptide, which is composed of a prodomain and a mature domain, belonging to the TGF-β superfamily. Several expression systems have been used to produce cost-effective and biologically active BMP-2 for therapeutic purposes. For instance, biologically active rhBMP-2 has been produced in CHO cells (Israel et al., 1992). Unluckily, the expensive medium requirements, sophisticated lab, and poor yield limit the use of CHO cells. Other studies used transgenic tobacco as a bioreactor for the production of rhBMP-2 (Suo et al., 2006). Yet extended duration is required for the growth of tobacco along with laborious purification protocols. In the production of biopharmaceuticals especially, when posttranslational modifications do not matter; e.g. in BMP-2, then *E. coli* is the method of choice. Yet for dimerization of BMP-2, the formation of disulfide bond is critical (Scheufler et al., 1999). Additionally, *E. coli* expressed rhBMP-2 in the form of inclusion bodies,

thereby, entailing in vitro refolding strategies (Mollania et al., 2013, Wang et al., 2013). On the other hand, although yeast is a lower eukaryote, but its glycosylation pattern differs from human, e.g. containing more mannose residues and are relatively antigenic if injected to the blood stream (Gerngross, 2004).

To overcome the current limitations of the existing expression systems and devise new expression hosts, a number of parasites genomes have been sequenced and published. Deep understanding of parasites genomics and proteomics, led to the development of tailored heterologous expression system. An example is the *L. tarentolae,* a protozoan parasite of gecko (*Tarentola annularis*). An interesting feature of this expression host is that it integrates in itself both the features of prokaryotes and eukaryotes. In addition, it has the protein folding and posttranslational modification machinery and has the ability to produce human like glycosylated heterologous proteins (Basile and Peticca, 2009).For example, biologically active and fully glycosylated human erythropoietin and human laminin-332 have been produced in *L. tarentolae* (Sugino and Niimi, 2012, Breitling et al., 2002). In one other study the expression of soluble amyloid precursor protein alpha with human type N- and O-glycosylation has also been reported. Biological activity of *L. tarentolae* derived soluble amyloid precursor proteins alpha was compared with those derived from mammalian cells. Comparative studies demonstrated no significant difference in biological activity (Klatt et al., 2013).

Based on the above evidences, we also used *L. tarentolae* for the expression of BMP-2. In the current study, we successfully cloned and expressed proBMP-2 gene in *L. tarentolae*. Molecular investigation of mRNA and chromosomal DNA revealed the successful integration proBMP-2 gene in *L. tarentolae ssu* locus. The expression was analyzed by employing SDS-PAGE and Western blotting. One single band of 12kDa was observed which could be attributed to the monomeric form of BMP-2. However, we did not observe the band of dimeric and mature BMP-2. The absence of dimeric form of BMP-2 could be due to its sensitivity to physicochemical conditions and improper handling, critical for maintenance of its structure and biological activity (Kisiel et al., 2012). Likewise, published studies have shown that several factors including, protein concentration, temperature, redox conditions, and the presence of stabilizing and destabilizing agents affects the folding and dimerization of BMP-2. Under all reported conditions the process of dimerization is very slow (Vallejo and Rinas, 2013). The absence dimer could be due to the last disulfide bond, responsible for dimerization, has been broken by autoprotease

activity, improper handling or extraction procedures. Above discussion demonstrated that the absence of dimerization of BMP-2 was not limited by *L. tarentolae*; rather, it needs controlled conditions for dimerization. We suggest further studies for the optimization of the physicochemical conditions in order to recover the dimerization.

Conclusion

L. tarentolae was able to express monomeric form rhBMPs. We anticipate that *L. tarentolae* is comparatively ideal candidate for the production of biologically active BMP-2, utilizing inexpensive medium.

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