MOLECULAR CLONING OF PARTIAL AND FULL LENGTH OF HUMAN PERIOSTIN GENE INTO EXPRESSION CONSTRUCT

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Molecular Cloning of Partial and Full Length of Human *Periostin* Gene into Expression Construct

Aziana Binti Abu Hassan

This project is submitted in partially fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
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Molecular Cloning of Partial and Full Length of Human Periostin Gene into Expression Construct

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ABSTRACT

Periostin which originally named as the osteoblast-specific factor 2 is highly homologous to βigH3, a molecule induced by transforming growth factor (TGF) - β1; and fasciclin 1 an insect adhesion molecule. Periostin was proved to play an essential role in promoting the growth of tumor cells. Functional studies of human periostin should be performed to intensively understand the function and expression of periostin in normal and cancer tissues. In this report, cDNA fragments of periostin from human normal colon total RNA are isolated by RT-PCR for the purpose of amplification of the gene fragments, clone into pTarget™ Mammalian Expression Vector System and sent to DNA sequencing analysis. Information generated from DNA sequencing analysis is used to confirm the successful construction of the periostin into expression vector. Expression constructed of periostin into pTarget™ vector can be used to begin functional studies of periostin inside mammalian cell lines system. The partial fragment of periostin gene with a predicted size of 685 bp was successfully amplified. However subcloned of periostin in pTarget™ vector was unsuccessful due to several problems. Therefore, DNA sequencing analysis was not perform.

Key words: periostin gene, pTarget™ Mammalian Expression Vector System, Reverse Transcription Polymerase Chain Reaction (RT- PCR), normal colon tissue, total RNA.

ABSTRAK


Kata kunci: gen periostin, pTarget™ Mammalian Expression Vector System, Reverse Transcription Polymerase Chain Reaction (RT- PCR), tisu usus normal, keseluruhan RNA.
<table>
<thead>
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<tr>
<td>AGE</td>
<td>Agarose Gel Electrophoresis</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Moloney- Murine Leukemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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ACKNOWLEDGEMENT

First of all I would like to send my appreciation to those persons who have gave me a lot of knowledge and guidance while I was finishing my final year project in Human Molecular Genetics Lab. I would like to send my highest appreciation for Dr. Edmund Sim Ui Hang who is my project supervisor. Special thank you for all Resource Biotechnology master students especially those who were in the Human Molecular Genetics Lab. Also for all my colleagues who were very helpful and understanding.
1.0 INTRODUCTION

Molecular cloning of partial and full-length human periostin into expression vector is a research to study the stability of periostin when being purified in an isolated environment and its compatibility to be constructed into an expression vector for further expression study of the characterized expressed protein of periostin.

Periostin is an extracellular matrix protein, originally named as the osteoblast-specific factor 2. Periostin is highly homologous to βigH3, a molecule induced by transforming growth factor (TGF)-β1, which promotes the adhesion and spreading of fibroblasts (LeBaron et al., 1995). It also has homology to fasciclin I, an insect adhesion molecule, which suggests involvement in cell adhesion (Horiuchi et al., 1999). Periostin was proved to play an essential role in promoting the growth of tumor cells. More recently, reports have implicated periostin in heart valve morphogenesis (Norris et al., 2004), in oncogenesis (Yoshioka et al., 2002) and in vascular smooth muscle cells (Lindner et al., 2005). The broad tissue distribution of periostin expression suggested that it has more generalized functions that are not limited to bone formation. Further in vitro and in vivo research of periostin expression is important to understand the variability functions of these proteins.

Molecular cloning technique was used in this project for accomplishing DNA manipulation for a variety of purposes. This method allows the gene of interest to be inserted and express in the expression vector. It is a useful technique to clone the
periostin gene into a pTarget™ Mammalian Expression Vector System and multiply it in the host cells. Therefore the expression and function of this gene can be studied in in vitro condition.

The expression construct is defined as the expression vector containing the coding sequence of the gene of interest. Segments of the expression construct should be analyzed to ensure the correct coding sequence has been incorporated into the expression vector. In this project, the isolated recombinant plasmid was digested with universal restriction endonucleases and verified by AGE in attempts to analyze the nucleic acid sequence.

The objective of this project is to generate periostin expression vector system which will facilitate in further study of the periostin protein within mammalian cells line. The hypothesis is to enable the purified periostin gene being carried inside the mammalian expression vector. This project studied the activity of periostin gene in the mammalian expression vector to determine whether the gene is suitable to be constructed into the mammalian expression vector. Basically this research will create a system, which enables the periostin to be transfected into host cells such as mammalian cell lines via mammalian expression vector and being expressed inside the cells. This would allow the study of the periostin gene in the isolated environment rather in in vivo. Results from this project will assist in future periostin expression study in normal and cancer cells especially in colorectal cancer research in UNIMAS.
2.0 LITERATURE REVIEW

2.1 Periostin gene

*Periostin* is a gene whose sequence is highly conserved between mice and humans (referred to Appendix A). *Periostin* contains an N-terminal secretory signal peptide, followed by a cysteine-rich domain, four internal homologous repeats and a C-terminal hydrophilic domain (Appendix A). Based on the data provided by Online Mendelian Inheritance In Man (OMIM), the International Radiation Hybrid Mapping Consortium mapped the human *periostin* gene to chromosome 13 which is located at 13q13.3. Figure 1 and 2 showed the genomic context of *periostin* gene mapped to chromosome 13.

![Figure 1: The Homo sapiens (human) genome view. The periostin gene was mapped to chromosome 13, located at 13q13.3. This diagram was obtained from National Centre of Biotechnology Information (NCBI) Map Viewer.](image-url)
Figure 2: The coding and untranslated region of periostin, located at 37070874 bp – 37034779 bp on chromosome 13 which shown on reverse complement genome. This diagram was obtained from National Centre of Biotechnology Information (NCBI) Entrez Gene.

*Periostin* is a putative bone extracellular matrix (ECM) protein previously named osteoblast – specific factor-2 (Li *et al.*, 2004). It has been suggested to function as a cell adhesion molecule for preosteoblasts and to participate in osteoblast recruitment, attachment and spreading (Horiuchi *et al.*, 1999). Studies by Sasaki *et al.* (2002) demonstrated that *periostin* was not osteoblast specific.

*Periostin* is highly homologous to *βigH3*, a molecule induced by transforming growth factor (TGF)-β1, which promotes the adhesion and spreading of fibroblasts (LeBaron *et al.*, 1995). It also has homology to *fasciclin I*, an insect adhesion molecule, which suggests involvement in cell adhesion (Horiuchi *et al.*, 1999). Based on its sequence homology to insect adhesion molecule *fasciclin I*, *periostin* may play a role in the adhesion process (Sasaki *et al.*, 2002). This has already been demonstrated in the mouse, because recombinant *periostin* supports the attachment and spreading of MC3T3-E1 cells in a solid-phase binding assay.

Related isoforms in the *periostin* family contains four domains of approximately 150 amino acids, referred to as repeat domains (Appendix A). The four internal repeats region of *periostin* shares a homology with the axon guidance protein *fasciclin I* that is involved
in the development of nervous system in invertebrates (Zinn et al., 1988). Structurally, within each repeat domain exists a conserved *fas* domain (Litvin et al., 2006). The four *Fas I* domains are postulated to be a binding domain for integrin from the results of biochemical analysis of β1gH3 (Kim et al., 2000). Studies by Litvin et al. (2006) implicated that periostin-like factor (PLF) which is one of the *periostin* isoform, and other isoforms of *periostin* have a potential as therapeutic targets.

*Periostin* is intimately associated with the progression of tumor development. The expression of *periostin* is up-regulated in various types of human cancers in comparison to normal tissue. *Periostin* is overexpressed in several human tumors including primary colon cancer, metastatic liver cancer from colon, and breast cancer (Bao et al., 2004). Tumor angiogenesis is an important process in tumor development in which the tumor attempts to gain an independent blood supply. Based on research by Shao et al. (2004) *periostin* was identified as a novel angiogenic factor that acts to promote tumor growth by inducing tumor angiogenesis. Vascular endothelial growth factor (VEGF) and its receptor Flk-1/KDR have been extensively documented to be involve in the induction of angiogenesis during the development of solid tumors (Shao et al., 2004). *Periostin* acts to increase the expression of the VEGF receptor (Flk-1/KDR), which in turns renders the cells more sensitive to the action of VEGF. Therefore, *periostin* was responsible as protein-mediated factor in tumor angiogenesis. As evidence, a study to compare level of hemorrhage between tumors derived from *periostin*-producing cells with control cells in human breast cancer, showed high levels of hemorrhage were associated with the tumors derived from the *periostin*-producing cells (Shao et al., 2004).
Metastatic process involved in later stage development of tumors. Metastases, rather than primary tumors, are responsible for most cancer deaths. Studies by Bao et al. (2004) reported that acquired expression of *periostin* by colon cancer cells greatly promoted metastatic development of colon tumors. *Periostin* expression dramatically enhanced metastatic growth of colon cancer by both preventing stress-induced apoptosis in the cancer cells and augmenting endothelial cell survival to promote angiogenesis. The mechanism of *periostin*-mediated promotion of tumor metastasis is largely associated with the ability of *periostin* to enhance cellular survival for both cancer cells and endothelial cells under stress conditions (Bao et al., 2004). *Periostin*-producing cells were more resistant under stress conditions that are commonly associated with the metastatic tumor and fast-growing tumor mass, such as hypoxia, nutrient depletion, and loss of adhesion. At the molecular level, *periostin* promotes cellular survival by activated the Akt/PKB pathway via the αvβ3 integrins. Binding of *periostin* to the αvβ3 integrins leads to activation of the Akt/PKB cellular survival pathway, consequently protecting both tumor cells and endothelial cells from stress-induced cell death and promoting angiogenesis and metastasis (Bao et al., 2004). As an angiogenic factor, *periostin* induced neovascularization by augmentation of the survival endothelial cells. Formations of new blood vessels in tumor derived from *periostin*-producing cells provide tumor cells with nutrients and oxygen under stress condition. Studies by Shao et al. (2004) reported that the hemoglobin content in tumors derived from *periostin*-producing cells was on average 30% higher than that in tumors derived from control cells. Therefore, *periostin* is important in inducing the tumor angiogenesis which is the critical aspects that allowed micrometastases to progress. The intimately association of *periostin* with tumors
proliferation and progression has brought to the idea of these proteins could be a potential target for blocking the growth of metastatic cancers.

Studies by Lindner et al. (2005) have found that expression of *periostin* also induced by vascular injury because the *periostin* expression is increased after arterial injury. Identification of cells expressing *periostin* mRNA within the vessel wall after injury was performed by *in situ* hybridization with an antisense *periostin* riboprobe. Low levels of *periostin* expression were detectable in normal arteries. After eight days of injury, high levels of *periostin* mRNA expression were observed in the adventitial fibroblast. The study indicated that *periostin* is associated with smooth muscle cells (SMC) differentiation and function as a migratory stimulus *in vitro*. After ballon catheter injury, *periostin* expression coincides with the proliferation phase of SMCs in the neointima (Lindner et al., 2005).

The *periostin* mRNA was also expressed in the developing mouse embryonic and fetal heart and that it is localized to the endocardial cushions that ultimately divide the primitive heart tube into a four-chambered heart (Kruzynska-Frejtag et al., 2001). Analysis with use of semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) indicates that *periostin* mRNA can initially detected within the isolated embryonic mouse heart at low level and continues to be strongly expressed throughout cardiovascular development and in the adult heart. Strong *periostin* expression could be detected by *in-situ* hybridization within the enlarging outflow tract and atrioventricular endocardial cushions, which are required for the septation and remodeling of the tubular
heart. Studies by Kruzynska-Frejtag et al. (2001) proved that expression of \textit{periostin} was essential in the developing mouse heart at sites that divide the primitive heart into the mature four-chambered heart. Additionally, \textit{periostin} is a useful marker of mesenchymal cells that have undergone epithelial-mesenchymal transformation within the developing heart (Kruzynska-Frejtag et al., 2001).

In addition, studies by Shao \textit{et al.} (2004) revealed that \textit{periostin} may confer an advantage for the growth of breast tumors \textit{in vivo} by altering the microenvironment through the induction of angiogenesis, its overexpression may impose a growth disadvantage when the tumor cells are grown in culture. These observations reveal the vital importance of relying on evidence derived from primary human cancer samples rather than established cell lines to draw major conclusion on the mechanisms and involvement of specific genes in tumorigenesis (Shao \textit{et al.}, 2004). Furthermore, the tumor-promoting effect of molecules such as \textit{periostin} can only be revealed by \textit{in vivo} analysis in animal models, rather than solely by \textit{in vitro} studies in cell culture (Shao \textit{et al.}, 2004).

This indicate that the result from the analysis of \textit{periostin in vitro} with use of engineered cell lines to produced \textit{periostin} in culture may different from the analysis of the \textit{periostin in vivo} cells derived from primary human cancer samples. Therefore, \textit{in vitro} study of \textit{periostin} is essential in order to understand this phenomenon and reveal \textit{periostin} functional mechanism in cell lines.
2.2 Mammalian Expression System

Studies in protein functional required the isolation a gene of interest and placing it in a controlled system for expression. Recombinants proteins can be expressed in several different systems, including bacterial and mammalian cells and even cell free systems.

Regulating recombinant protein expression in mammalian cells has become an integral part of protein production and analysis. Having the ability to control when and how much a particular protein is expressed makes it possible to study temporal- and concentration-dependent effects on a host system and to express toxic proteins. Studying the host cell response to the presence or absence of a protein often leads to further understanding of that protein’s function.

Mammalian cells offer several advantages over prokaryotic cells for gene expression. Proteins from eukaryotic organisms that are produced in mammalian or other eukaryotic systems are more likely to be functional as the processes of transcription, translation and posttranscriptional modifications are conserved.

Mammalian expression system has been developing to overcome the limitation in expression of mammalian recombinant proteins in E. coli system. Many mammalian recombinants proteins are poorly expressed in E. coli due to a number of technical limitations, including poor solubility and inefficient translation due to codon bias. Furthermore, many mammalian proteins undergo functionally relevant post-translational modifications that are not carried out in E. coli (Geng and Carstens, 2006). Mammalian
cell lines that are commonly used include the 293 cell line and its variants, COS cells and the Chinese hamster ovary (CHO). The 293 cell line is a popular cell line for expressing mammalian proteins due to high transfection rates and efficient expression from commonly used eukaryotic promoters such as that derived from cytomegalovirus (CMV) (Liu et al., 1997).

Studies by Iida et al. (2005) in constructed expression system for the motor protein prestin in Chinese hamster ovary (CHO) cells, has used three types of expression systems in attempts to produced large amount of prestin to obtained knowledge on the function of prestin at the molecular level. The expression system that have been utilized were E. coli expression system with use of E. coli expression vector such as pET28b, pET20b and pMAL-c2, baculovirus expression system with use of pVL1392 baculovirus expression vector, and mammalian expression system with use of pIRES-hrGFP-1a mammalian expression vector. Out of these three expression systems, the pIRES-hrGFP-1a mammalian expression vector system which carried prestin cDNA and transfected into CHO cells showed high degree of prestin expression in CHO cells. In CHO cell expression system a strong, broad band around 90 kDa was detected in transfected cells, and bands around 30 kDa were detected in both transfected cells and untransfected cells. These results indicate that the prestin is expressed well in CHO cells (Iida et al., 2005).

Transient mammalian expression system such as episomal expression vectors offer a faster alternative and have been used to transfect HEK293-EBNA cells in suspension and produced up to 50 mg/L of protein in bioreactors in a matter of days (Meissner et al., 2001). Episomal vectors that are commonly used include pEAK8 (Edge Biosystems) and
pCEP4 (Invitrogen). Aside from the advantages of rapid and high levels of expression, multiple episomal vectors containing gene constructs each with a different selection marker may be transfected into the same pool of cells (Horlick et al., 2000). This may be advantageous where the protein exists as a heterodimer, or where co-expression is required to express soluble active protein (Davies et al., 2005).

Studies by Bao et al. (2004) have constructed a periostin expression plasmid by cloned the full open reading frame of human periostin cDNA into pcDNA3.1 mammalian expression vector (Invitrogen) with a hexa-histidine tag at COOH terminus of periostin protein. The periostin/pcDNA3.1 was introduced into CX- INS colon cancer cells, and the stable clones were obtained by G418 selection (950 μg/ml) in the culture medium.

In addition, studies by Plesa et al. (2003) have used pTargetT™ (Appendix B) mammalian expression vector system in assessing the antibody response against HPVs (Human Papillomavirus) in murine cells by the expression of the L1 major capsid viral protein type 18 (HPV 18) into L929 murine cells. The pTargetT™ vector design allows only in-frame orientation of the insert and the 1706 bp fragment was used as such for insertion into the vector at a molar ratio insert: vector, 10: 1 (for 60 ng vector, 540 ng amplicon was added) (Plesa et al., 2003). The pTargetT™ is a linear vector, its present 3'T overhangs into the split lacZ gene MCS (Multiple Cloning Site) and the HPV 18 amplicon was ligated into to the vector using the T4 DNA ligase, as recommended by the Promega protocol (Plesa et al., 2003). The pTargetT™ vector is based on the pCI-neo Mammalian Expression Vector but the principle differences between pTargetT™ and the
pCI-neo vector are the presence of the alpha-peptide coding region of lacZ (which allows for blue/white screening on indicator plates) and the single- T overhangs of the cloning site.

Those studies indicate that mammalian expression system is the most compatible system for protein expression. There were varieties of mammalian expression vector which commercially available. In this project the pTargetT™ mammalian expression vector was used to construct the expression system for perioistin cDNA. However, the transfection of perioistin/ pTargetT™ into mammalian cell lines will be done in further analysis of perioistin protein. This study was performed to generate an expression system for perioistin cDNA which will assist in vitro study of human perioistin protein in mammalian cell lines such as 293 cell line and its variants, COS cells and the Chinese hamster ovary cells (CHO).
2.3 RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) cDNA Synthesis

Original genes of eukaryotic organisms contain introns which can interfere proteins expression. The introns do not code for any amino acids sequences and separate the genes within the exons which are the coding sequences for protein synthesis. To remove the introns the genes undergone RNA processing in which the intron is looped out and cut away from the exons by snRNAs (single nucleotide RNAs) and the exon are spliced together to produce the complete mRNA, leaving only the coding sequence. The mRNA cannot be cloned directly but a DNA which is a copy of the mRNA in terms of cDNA, can be cloned (McClean, 1997). In order to measure mRNA, the method was extended using RT to convert mRNA into cDNA which was then amplified by PCR and, analyzed by AGE (Nair-Menon and Li, 2005). Reverse transcriptase synthesizes a DNA strand which is complement with the mRNA template if it is provided with a primer that is based paired to the RNA and contains a free 3'-OH group. Usually this enzyme used to synthesize DNA from mRNA by providing an oligo-dT primer that pairs with the poly-A sequence at the 3’ end of most eukaryotic mRNA molecules (Stryer, 1989). The synthesize of the first strand of cDNA is accomplished by the presence of the four deoxynucleotides (dATP, dGTP, dTTP, dCTP), RNase H which degrade the mRNA template into small fragments and become the primer and DNA polymerase I for allowing the second strand of cDNA to be formed. A PCR mix is then set up which includes a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides and a suitable buffer (Nair-Menon and Li, 2005). The purpose of doing RT-PCR in this project is to isolate the periostin cDNA from the human normal colon total RNA.
3.0 MATERIALS AND METHODS

3.1 Sampling sources
The sample was commercially-available human normal colon total RNA provided by post-graduated student (Ma Xiang Ru, Human Molecular Genetic Lab, UNIMAS).

3.2 Isolation of Periostin cDNA

3.2.1 Synthesis of the first strand cDNA
The periostin cDNA was isolated using RT-PCR method. (M-MLV RT) (catalog # M 1701, Promega, USA) was used to obtain the first complementary single-stranded cDNA. M-MLV RT is an RNA- dependent DNA polymerase that can be used in cDNA synthesis with long messenger RNA templates. The RNase activity of M-MLV RT is weaker than the commonly used Avian Myeoblastosis Virus (AMV) reverse transcriptase. For first strand cDNA synthesis, the following components were mixed in the order below:

- 1 µl 50-250 ng of Oligo (dT) primer
- 1 µl of commercially available human normal colon total RNA
- 11.5 µl of Nuclease-free water

Then the mixture was heated at 70°C for 10 minutes and the tube was immediately cooled on ice to prevent secondary structure from reforming. Then, the tube was centrifuged briefly. After that the following components were added:

- 25 Mm dNTPs mix 1 µl
- M-MLV RT 5x Reaction Buffer 4 µl
- RNase Inhibitor 0.5 µl
The tube was mixed gently by flicking of the tube and it was incubated at 42°C for 2 minutes. After incubation, 1 µl of M-MLV RT was added and gently mixed it by pipetting. The final volume of the RT reaction mixture was 20 µl. The mixture was incubated at 42°C for 50 minutes. Next, the reaction mixture was being inactivated at 70°C for 15 minutes. The reaction mixture was stored in the refrigerator at -20°C and ready for the next usage in PCR reaction as a template for isolation and amplification of periostin gene.

3.2.2 Synthesize of the second strand cDNA

The reaction mixture of first strand cDNA was used as the template for PCR in the second strand cDNA synthesis. Thermostable DNA polymerase from Thermus aquaticus or Thermus thermophilus synthesized the second strand of cDNA. For each PCR cycle, components as stated in Table 1 were added in the 0.2 ml microcentrifuge tube:

| Table 1: Ingredients and their appropriate amount used in each PCR reaction |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Components                  | Stock Concentration | Final Concentration | Volume (µl)  |
| PCR Buffers                 | 10x              | 1x               | 2.5           |
| MgCl₂                       | 25 mM            | 1 mM             | 1             |
| dNTP mix                    | 10 mM            | 0.2 mM           | 0.5           |
| OSF-2p1-F1                  | 25 pmol/µl       | 1 pmol/µl        | 1             |
| OSF-2p1-R1/R4               | 25 pmol/µl       | 1 pmol/µl        | 1             |
| Fermentas Taq DNA Polymerase| 5 U/µl           | 0.02 U/µl        | 0.1           |
For isolating the *periostin* gene, the primers that were used in this study were provided by Research Biolabs, Singapore and were utilized in every PCR reaction. Table 2 and Table 3 showed the nucleotides sequence for each primer.

Table 2: Primers that were used in PCR amplification in attempts to isolate partial length of human *periostin* gene

<table>
<thead>
<tr>
<th>Primers Sequence</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSF-2p1-F1</td>
<td>5' AACTGCAACGGAGAGACTCAA 3'</td>
</tr>
<tr>
<td>OSF-2p1-R1</td>
<td>5' CACCATTTGTTGCAATCTGG 3'</td>
</tr>
</tbody>
</table>

Table 3: Primers that were used in PCR amplification in attempts to isolate full length of human *periostin* gene

<table>
<thead>
<tr>
<th>Primers Sequence</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSF-2p1-F1</td>
<td>5' AACTGCAACGGAGAGACTCAA 3'</td>
</tr>
<tr>
<td>OSF-2p1-R4</td>
<td>5' CCACAATTTCCTCATGTTTC 3'</td>
</tr>
</tbody>
</table>

In every PCR amplification, one negative control was used to monitor the validity of the PCR reaction.

The followed table (Table 4 and Table 5) will showed the parameters for the PCR reactions in attempts to isolate the full and partial of human *periostin* cDNA.