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## Effect of BMP Treatment on Periostin Gene Expression in Pre-Osteoblastic MC3T3-E1 Mouse Cells

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EFFECT OF BMP TREATMENT ON PERIOSTIN GENE EXPRESSION IN  
PRE-OSTEOBLASTIC MC3T3-E1 MOUSE CELLS

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A thesis submitted in partial fulfillment  
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## **Thesis Approval**

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Effect of Bmp Treatment on Periostin Gene Expression in Pre-Osteoblastic MC3T3-E1  
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## Abstract

Periostin is a secreted, extracellular matrix (ECM) protein widely expressed within collagen-rich fibrous connective tissues of the body including the periodontal ligament (PDL), bone, skin, heart, and cornea. Periostin has been shown to serve many important regulatory functions including cell adhesion, cell motility, wound healing and of particular importance to the dental field, differentiation of osteoblasts. The deletion of periostin compromises osteoblast attachment to bone matrix and induces a reduction in mineralization and expression of bone markers, including type I collagen, osteocalcin, osteopontin and alkaline phosphatase. Periostin has also been shown to play a significant role in collagen fibrillogenesis by enhancing the proteolytic activation of lysyl oxidase, which is required for collagen cross-linking.

Immunohistochemistry studies have revealed high levels of periostin expression in the PDL with the periostin expressing cells identified as the fibroblastic cells in the PDL and osteoblastic cells on the alveolar bone surfaces. The significance of periostin to bone and PDL development was further demonstrated in a study on periostin knockout mice. These mice displayed a unique phenotype with significant changes to the periodontium: gingival tissue atrophy, PDL damage and loss of bone around the molars, underscoring the importance of periostin as a key ECM protein within the PDL. In controlling osteoblast differentiation and collagen fibrillogenesis, periostin has a critical role in maintaining bony architecture and density, which suggests that it may be able to act as another layer of therapeutic control on bone homeostasis.

Cytokines of the transforming growth factor-beta (TGF- $\beta$ ) superfamily of proteins including TGF- $\beta$ 1 have been implicated in the regulation of periostin expression in bone. In particular, studies have shown that periostin expression is enhanced with increasing levels of

TGF- $\beta$ 1 until bone mineralization begins. At that point, periostin expression is negatively regulated as mineralization proceeds, suggesting that TGF- $\beta$ 1 may play a role during the initiation of bone mineralization. Another member of the TGF- $\beta$  superfamily, bone morphogenetic protein 2 (BMP2), has also been demonstrated to enhance periostin expression in osteoblasts. Recently, there has been increasing interest in bone morphogenetic proteins (BMPs) due to their therapeutic potential in orthopedics, oral surgery and other disciplines. BMPs are members of the TGF- $\beta$  superfamily of proteins and act as regulators during embryogenesis and bone and cartilage formation and repair. Research in the area of BMP action has revealed great complexity with far reaching effects among the many members of the various BMPs. Although periostin expression in pre-osteoblastic cells, specifically MC3T3-E1 mouse pre-osteoblasts, has been studied in response to TGF- $\beta$ 1 and BMP2, other BMP members have not been considered. Given that different BMP family members are differentially expressed in tissues of the body with various physiological functions, it is reasonable to assume that they may have different effects on periostin expression as well. For example, BMP2, BMP4 and BMP7 all play key roles in bone and cartilage development whereas BMP3 has been characterized as an antagonist to the osteogenic effects of the other BMPs.

The objective of this study was to demonstrate the *in vitro* expression of periostin in MC3T3-E1 mouse pre-osteoblast cells in response to different BMPs. Previous studies describing the regulation of periostin expression by TGF- $\beta$ 1 suggests that periostin has the potential to be a downstream effector of the TGF- $\beta$  superfamily of proteins. In this study, the expression of periostin was hypothesized to increase with BMP2, BMP4 and BMP7 treatment, supporting the notion of these BMPs as enhancers or agonists of periostin expression. In contrast, BMP3 was hypothesized to suppress periostin expression due to its innate inhibitory potential.

However, BMP3 does possess modulator activity and could also enhance periostin expression under some conditions as well. Unlike other studies, this research is unique in attempting to determine not only the effects of BMP2, but also BMP4 and BMP7 on periostin expression, which, to our knowledge, have never been considered. In addition, no previous studies have considered the antagonistic effects of BMP3. As a way to further analyze this relationship, the effect of concentration was to be considered as well. Ultimately, understanding the effects of BMPs on periostin expression will contribute to our overall understanding of the complex mechanisms involved in maintaining osteoblasts in an undifferentiated state as well as their therapeutic applications in the clinical setting. In the future, this knowledge may have important clinical implications in the modulation of osteoblast activity, which may be applicable to the dental field in the regulation of tooth movement, regeneration of the periodontium and *de novo* bone formation.

MC3T3-E1 pre-osteoblast cells were prepared and treated in duplicate with BMP2, BMP3, BMP4 and BMP7 with two concentrations: 10 ng/mL and 25 ng/mL. After 24 hours of incubation under controlled conditions, cells were lysed and total RNA was purified, extracted and stored at -80°C. Reverse transcription polymerase chain reaction (RT-PCR) was performed on all samples using periostin primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. These particular primers were selected to amplify a region of periostin cDNA as well as the typical housekeeping protein (GAPDH) as a normalization factor for cell number. Amplified products were run on a 2% agarose gel for two hours followed by visualization and image capture under a UV light. Expected products were identified against the known base pair values of periostin and GAPDH. Pixel density was quantified for each band and periostin bands were normalized against their corresponding GAPDH bands. Results indicated that periostin

expression was significantly increased in both BMP2 and BMP3 treatments. The following increases in periostin expression from baseline were observed: BMP2 (10 ng/mL): +29%, BMP2 (25 ng/mL): +26%, BMP3 (10 ng/mL): +24% and BMP3 (25 ng/mL): +17%. Periostin expression was also increased under BMP4 (+9% and +11% for 10 ng/mL and 25 ng/mL concentrations respectively) and BMP7 (+5% and +11% for 10 ng/mL and 25 ng/mL concentrations respectively) conditions, although this was not statistically significant. These findings confirm the observation from other studies that BMP2 enhanced periostin expression. However, BMP3 showed contrasting results and actually increased periostin expression, suggesting a modulator role for BMP3 on periostin expression. BMP4 and BMP7 did not elicit significant changes on periostin expression, which may be due to a number of factors. Concentration-dependence was not observed for any of the BMPs. Future studies are needed to further evaluate the relationship between periostin expression and BMPs.



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## Preface

In the field of molecular proteomics, periostin is considered to be a relatively novel protein. Previously known as osteoblast-specific factor 2 (osf-2), periostin is a secretory protein originally isolated in the periosteum and periodontal ligament (PDL) of mouse cells and renamed shortly thereafter. Horiuchi *et al.* (1999), the researchers who first isolated and renamed periostin, correctly hypothesized that periostin must have a role in regulating the functions and processes of bone formation and PDL metabolism due to its physical proximity to both of these structures (Horiuchi *et al.*, 1999). Results from their study demonstrated periostin's role in cell adhesion and osteoblast-like cell recruitment and a differential expression pattern (Horiuchi *et al.*, 1999). Conclusions derived from their experiments ultimately put periostin on the scientific map and garnered significant interest from the scientific community including researchers from disciplines outside of bone biology. Since then, other studies have shown that periostin's expression is, in fact, ubiquitous throughout the body with a particular localization in collagen-rich fibrous connective tissues (Nicolas Bonnet, Garnero, & Ferrari, 2016; Horiuchi *et al.*, 1999; Shimazaki *et al.*, 2008). This type of connective tissue functions to maintain the structural integrity of various organ systems subject to daily mechanical stresses, suggesting that periostin may have important implications in maintaining these structures (Horiuchi *et al.*, 1999). Periostin has also been demonstrated to have key roles in early development of the heart, teeth and periodontium (Hakuno *et al.*, 2010; Lindner, Wang, Conley, Friesel, & Vary, 2005). Other studies have identified periostin during pathological states including cancer, cardiovascular injury, bone disease and wound healing (Kashima *et al.*, 2009; Lindner *et al.*, 2005; Otsuka *et al.*, 2012; Ruan, Bao, & Ouyang, 2009). Collectively, it is clear that periostin has a wide array of roles throughout the body and is an important player for physiologic homeostasis.

Of particular interest to dental professionals and researchers is the periodontium. The periodontium, also known as the periodontal apparatus, is a highly organized structure that maintains and supports the dentition within the maxillary and mandibular dental bases using a molecular backbone of fibrous connective tissue. It consists of four key elements: the PDL, alveolar bone, cementum and gingiva (Padial-Molina et al., 2012). Not only does it act as a foundation for the teeth by attaching them to the bones of the jaws, the periodontium also plays a role in proprioception, nociception and cushioning impact during mastication (Padial-Molina et al., 2012). The integrity of the periodontium is absolutely vital in maintaining healthy support of the teeth and without it, teeth would become loose and eventually be lost. As it turns out, periostin is highly expressed in the PDL of the periodontium and shown to be involved in regulating bone metabolism in that region (Horiuchi et al., 1999; H. Rios et al., 2005). This fact raises two important questions: 1) Can periostin expression in the PDL be controlled or regulated? And if so, 2) does this level of control bring about any benefits on a clinical and patient care level? Previous research has shown that certain cytokines, small biologically active signaling proteins, play a role in regulating the expression of periostin (Ali & Brazil, 2014; Carreira et al., 2014; Oryan, Alidadi, Moshiri, & Bigham-Sadegh, 2014). The transforming growth factor beta (TGF- $\beta$ ) superfamily of proteins is a large group of regulatory proteins, which share certain structural similarities amongst each other due to a common homology (Ali & Brazil, 2014; Carreira et al., 2014; Oryan et al., 2014). These proteins have been examined extensively and been shown to have an effect on periostin expression; specifically, transforming growth factor beta 1 (TGF- $\beta$ 1) and bone morphogenetic protein 2 (BMP2) of the TGF- $\beta$  superfamily have been demonstrated to enhance the expression of periostin along an osteoblast cell line (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle, Bouet, Rousseau, Bertholon, &

Garnero, 2014). Despite similarities in structure, members of the TGF- $\beta$  superfamily carry surprisingly different functions and roles depending on where they are expressed in the body, which suggests a possible means of control of periostin expression. Extrapolating this notion would suggest that other proteins of the TGF- $\beta$  superfamily may not necessarily have the same effect on periostin expression, despite a shared homology.

Known members of the TGF- $\beta$  superfamily understood to play a role in bone and cartilage development include the following bone morphogenetic proteins (BMPs): BMP3, BMP4 and BMP7. To our knowledge, these proteins have never been studied in conjunction with periostin, a clear gap in the knowledge of this area. If these particular cytokines have a role in bone metabolism, perhaps they may have a regulatory function on periostin expression as well. The purpose of this research was to demonstrate whether BMP3, BMP4 and BMP7 have an effect on periostin expression and if so, whether this effect was stimulatory, inhibitory or inconclusive in nature. BMP2 will also be examined and was hypothesized to enhance periostin expression, as demonstrated on multiple occasions in the literature (Ji et al., 2000; Blandine Merle et al., 2014). The results from this study will be important to elucidate the layers of control governing periostin expression which may, in fact, cross over on a clinical level, not only in dentistry, but in other disciplines as well.

The following excerpts will provide a detailed review of the most current literature surrounding periostin and the different BMPs and along the way, further illuminate the importance of periostin as a research focal point. Pertinent background, relevant research studies as well as reasons for consideration will all be discussed in an in depth manner to ultimately



provide a global understanding of periostin and BMPs. Justification for the selection of the various BMPs involved in this study will become clearer through the literature review.

## Chapter 1: Introduction

### 1.1: Periostin

Takeshita *et al.* (1993) originally discovered periostin as osteoblast-specific factor 2 (osf-2) using a subtraction hybridization library from the cDNA of a MC3T3-E1 pre-osteoblast mouse cell line (Takeshita *et al.*, 1993). Using a similar technique, Horiuchi *et al.* (1999) delved further into the investigation and uncovered periostin's potential role in bone and tooth formation (Horiuchi *et al.*, 1999). Using a mouse probe, the human variant of periostin was discovered with a 90% homology to the mouse variant. Despite a shared homology, there are some differences between the two variants. For one, mouse periostin is located on chromosome 3 while the human variant is on chromosome 13, both consisting of 23 exons (Kudo, 2017). Mouse periostin is also slightly larger at 838 amino acids whereas human periostin is 836 amino acids in length (Kudo, 2017). The signaling peptide for the mouse and human variants are 24 and 22 amino acids long respectively (Kudo, 2017). Taking into account the signaling peptides which are post-translationally cleaved, the final product is 814 amino acids long for both types of periostin and thus, exactly the same in size and molecular weight. In its final form, periostin is a small, secreted, N-glycosylated protein at 90 kDa with a highly complex molecular structure. Its structure contains the secretory signaling peptide, cysteine-rich elastin microfibril interfacial-like (EMI) domain at the amino terminal (N-terminus), four internal fasciclin-1 (Fas-1) domains and a variable carboxy-terminal domain (CTD) (Horiuchi *et al.*, 1999; Kudo, 2011; Sugiura, Takamatsu, Kudo, & Amann, 1995). The EMI domain is a small module rich in cysteine residues that can interact with type I collagen, fibronectin and Notch1 whereas the Fas-1 domains have been demonstrated to interact with tenascin-C and BMP1 (Kii *et al.*, 2010; Maruhashi, Kii, Saito,

& Kudo, 2010; Tanabe et al., 2010). Indeed, periostin is classified as a member of the fasciclin family of proteins in having the highly conserved Fas-1 domains, which have been shown to be involved primarily in cell adhesion (Kim et al., 2000). Periostin's function in cell adhesion is likely due to its interactions with  $\alpha\beta3$  and  $\alpha\beta5$  integrins, transmembrane proteins responsible for facilitating cell-cell and cell-matrix interactions for cell migration and cell adhesion. Laminin  $\gamma2$  is another protein that interacts with periostin in a similar manner, although its exact purpose is still unknown (Conway et al., 2014; Kudo, 2011). The Fas-1 domains, rich in glutamate residues, act as the recognition and modification site for the enzyme,  $\gamma$ -glutamyl carboxylase (Coutu et al., 2008; Du & Li, 2017). This vitamin K-dependent enzyme post-translationally modifies the Fas-1 domain and converts the glutamate residue to  $\gamma$ -carboxyglutamate at one of the many carboxylation sites (Coutu et al., 2008; Du & Li, 2017). It is unclear whether this carboxylation exists in bone and affects the function of periostin, as it does for other bony proteins such as osteocalcin (Coutu et al., 2008). Cell adhesion sites within the Fas-1 domain allow periostin to interact with the above mentioned proteins, tenascin-C and BMP1 (Horiuchi et al., 1999; Kii et al., 2010). In a similar manner, the EMI domain within the N-terminus of periostin is responsible for protein-protein interactions. It is this particular site that interacts with the macromolecules, collagen type I, fibronectin and Notch1 (Kii, Nishiyama, & Kudo, 2016; Maruhashi et al., 2010; Morris et al., 2007). The EMI domain also has the potential to form disulfide-bonded dimers (Kii et al., 2010; B. Merle & Garnero, 2012; G. Takayama et al., 2006). The CTD is variable and contains four N-glycosylation sites and a heparin binding domain allowing it to bind to glycoproteins, glycosaminoglycans and proteoglycans (Sugiura et al., 1995). The CTD also possesses alternative splicing, which can produce at least five different human isoforms, adding yet another layer of structural complexity to this protein (Hoersch &

Andrade-Navarro, 2010b). In one instance, TGF- $\beta$ 1 treatment in mouse pre-osteoblast cells has been shown to produce splice variants, which increase in response to increasing TGF- $\beta$ 1 concentrations, suggesting that splice variants may be externally controlled with cytokines (Kudo, 2017). From its conserved structure, it can be seen that periostin has the potential to interact with a number of different proteins on many levels to help facilitate its functions. As a whole, the structural complexity and wide myriad of interactions that periostin facilitates suggests that this protein not only provides structural support, but also is involved in many different aspects of connective tissue differentiation, function and morphology (Cobo et al., 2016).

Alternative splicing of the CTD of periostin creates a number of periostin isoforms, which are differentially expressed in different tissue types, development stages and pathologies (B. Merle & Garnero, 2012). The CTD is encoded by exons 15 through 21 and identified by the corresponding six cassette exons, a through f. This nomenclature system was bestowed by Horiuchi *et al.* (1999) during the initial discovery of periostin (Horiuchi et al., 1999). Exon cassettes are added or deleted from the final periostin messenger RNA (mRNA), which gives rise to the different periostin isoforms (Horiuchi et al., 1999). For instance, periostin isoform 1 contains all six cassettes whereas periostin isoform 3 only has five cassettes as cassette e has been spliced out (Litvin et al., 2004). Another isoform missing both cassettes b and e has been shown to be highly expressed in the periosteum and PDL as well as during myocardial infarction, solidifying the notion of a differential expression pattern for each periostin isoform (Kudo, 2011; B. Merle & Garnero, 2012). Despite the fact that periostin is a known secreted protein, some isoforms of periostin such as periostin isoform 3 have been identified to carry a nucleus localization sequence within the CTD, which suggests that periostin may have extracellular as

well as intracellular roles (Kudo, 2011). Together, the splice variants of the CTD generate numerous periostin isoforms, each with a differential expression pattern. No matter the isoform, the CTD couples with the other domains of periostin, forming a complex, three-dimensional, functional protein. The proposed three-dimensional structure of periostin is as follows: four Fas-1 domains containing a secondary structure of helix-turn-helix motifs and the CTD with beta-strand structural elements (Coutu et al., 2008; Hoersch & Andrade-Navarro, 2010b; B. Merle & Garnero, 2012; Takeshita et al., 1993).

When periostin was first identified, it was initially thought to be specific to the periosteum of long bones and the PDL, hence the name. However, periostin is actually broadly expressed throughout the body within many tissue systems and by studying the areas with the highest levels of expression, the functions of periostin become more apparent. By employing cross-reactive antibodies to periostin, studies have demonstrated that periostin is primarily localized to the collagen-rich fibrous connective tissues of the body including the PDL, periosteum, aorta, stomach, lower gastrointestinal tract, placenta, uterus, thyroid tissue, cornea and breast (Nicolas Bonnet et al., 2016; Horiuchi et al., 1999; Shimazaki et al., 2008). Within the periosteum, osteoblasts have been shown to secrete periostin, which is involved during embryogenesis, bone repair and bone remodeling during mechanical stress (Nicolas Bonnet et al., 2009; Litvin et al., 2004). Periostin's role during osteoblast differentiation and collagen fibrillogenesis underscores its importance as a key regulator of bone and connective tissue microarchitecture and strength, which adds further evidence to the notion of periostin as a structural protein (Litvin et al., 2004; Maruhashi et al., 2010; Blandine Merle et al., 2014; H. Rios et al., 2005). The common underlying factor of all of the tissues where periostin is highly expressed is that they are all subject to mechanical stresses in one form or another through every

day physiologic functions, particularly the heart valves, PDL, tendons and bones (Nicolas Bonnet et al., 2016; Horiuchi et al., 1999). During physiologic homeostasis, periostin is involved in cell adhesion and cell migration through its Fas-1 domain interactions (Cobo et al., 2016; Kim et al., 2000). During unregulated periostin expression, physiology leads to pathology as the lack of control of cell adhesion and structural integrity results in compromised tissue systems. Abnormal periostin expression has been implicated in cases of myocardial infarction, respiratory diseases, fibrosis, wound healing and cancer-associated stroma (Fukushima, Kikuchi, Nishiyama, Kudo, & Fukayama, 2008; Kudo & Kii, 2017; Nishiyama et al., 2011; Okamoto et al., 2011; Shimazaki et al., 2008; G. Takayama et al., 2006). Taken together, this evidence suggests that periostin has a structural and regenerative capacity throughout the body during normal physiology, which can become pathologic in the absence of control.

While scientists have taken a significant interest in periostin since its discovery, much of the current literature on periostin's functions is scattered across many disciplines, fragmenting the overall understanding of this protein (Conway et al., 2014; Horiuchi et al., 1999). Still, extensive research on periostin has defined specific functions across many domains including osteology, periodontology, wound healing, oncology, cardiovascular and respiratory diseases and in various inflammatory states linked to cell adhesion and structural microarchitecture (Cobo et al., 2016; Lindner et al., 2005; Litvin et al., 2004; H. Rios et al., 2005; Tilman, Mattiussi, Brasseur, van Baren, & Decottignies, 2007). In a study by Morris *et al.* (2007), periostin knockout mice demonstrated a disruption in collagen fibrillogenesis within the periosteum and tendons resulting in an overall decrease in bone mass and bone strength (Morris et al., 2007). Not only were the bones affected, the skin dermis, a tissue packed with collagen, was also weakened. This reduction in strength across both tissues was attributed to aberrant collagen matrix

formation as a consequence of periostin absence, which ultimately had an inhibitory effect on bone remodeling and collagen formation and cross-linking (Morris et al., 2007). A reduction of mineralization and expression of key bone markers including type I collagen, osteocalcin, osteopontin and alkaline phosphatase was also noted in a different study (N Bonnet, Conway, & Ferrari, 2012; Litvin et al., 2004). A study by Kii *et al.* (2010) demonstrated similar findings in collagen fibrillogenesis in periostin knockout mice (Kii et al., 2010). Compared to the control group, periostin knockout mice developed tibial periostitis as a consequence of abnormal bridging between tenascin-C and the ECM, findings which suggest a key ECM bridging function for periostin (Kii et al., 2010). Periostin enhances the proteolytic activation of lysyl oxidase, which is a necessary step for collagen cross-linking (Kudo, 2011). Other studies have shown periostin acting as a scaffold and enhancing the intermolecular interactions between a number of ECM proteins and accessory proteins, processes necessary for the formation and organization of a cohesive ECM (Kii et al., 2010; Kudo & Kii, 2017). The interaction between periostin and the ECM proteins largely occurs via transmembrane  $\alpha\beta3$  and  $\alpha\beta5$  integrins, which is responsible for physiologic maintenance of tissue integrity under mechanical stress (Cobo et al., 2016).

Extrapolating these results to the PDL, another highly collagenous tissue, suggest a parallel relationship for periostin in mechanotransduction during masticatory loading and stress as well (Conway et al., 2014; Morris et al., 2007). Periostin's role in dental development and in particular, periodontology, is emphasized in another set of periostin knockout mice studies. In a study by Rios *et al.* (2005), periostin knockout mice were generated to investigate the role of periostin during dental development (H. Rios et al., 2005). The pervasiveness of periostin's involvement during development was immediately evident as 14% of periostin knockout mice died before weaning and the remaining mice experienced severe delays in growth (H. Rios et al.,

2005). Not only was dwarfism apparent, the remaining mice also demonstrated early-onset periodontal disease, gingival atrophy, bone loss around the molars, PDL damage and enamel defects, suggesting that periostin is critical to both dental development and reinforcing the integrity of the PDL (H. Rios et al., 2005). Periostin immunoreactivity at the bell and cap stages of tooth development, along the alveolar bone surface and within fibrous bundles of the PDL further confirm its involvement during dental development (Romanos, Asnani, Hingorani, & Deshmukh, 2014; H. Suzuki et al., 2004). Immunolocalization experiments by Suzuki *et al.* (2004) showed periostin expression restricted to the cytoplasmic extensions of immature fibroblasts only and not mature fibroblasts, which highlights the participation of periostin during development and remodeling of the PDL (H. Suzuki et al., 2004). Periostin also has a role in inducing bone formation through an increase in osteoblast differentiation and proliferation (S. Zhu et al., 2009). Overexpression of periostin resulted in greater mineralization and calcium deposition *in vitro* and *in vivo* when injected into a rat femur (S. Zhu et al., 2009). By controlling osteoblasts, periostin facilitates the bone remodeling process to enhance bone deposition and bone strength. Periostin expression is also increased during times of tissue repair as well as in some cancers. Studies have shown that periostin promotes wound healing by inducing the activation, differentiation and contraction of fibroblasts (Elliott et al., 2012; Nishiyama et al., 2011; Ontsuka et al., 2012). Increased expression of periostin was observed in the granulation tissues within the wounds of injured mice whereas in periostin knockout mice, wound repair and re-epithelialization was significantly impaired, all of which imply periostin's role in wound healing (Jackson-Boeters, Wen, & Hamilton, 2009; Nishiyama et al., 2011; Ontsuka et al., 2012). In oncology, the expression of periostin is up-regulated in certain cancers to promote tumor angiogenesis, migration and metastases as a direct consequence of malicious cell adhesion and



migration (Siriwardena et al., 2006). Periostin contributes to tumor development, angiogenesis and migration through its interactions with  $\alpha\beta3$  and  $\alpha\beta5$  integrins, promoting cell adhesion necessary for tumor growth and tumor cell motility (Cobo et al., 2016; Sasaki et al., 2003; Shao et al., 2004). A study by Bao *et al.* (2004) showed that a colon cancer cell line, having low metastatic potential initially, was able to develop enhanced metastatic potential upon being transduced to overexpress periostin (Bao et al., 2004). Cancer cell apoptosis was prevented and in fact, cancer cells were augmented to overgrow leading to angiogenesis (Bao et al., 2004). Overexpression of serum periostin in some cancers appears to be linked to an overall lower prognosis of survival, suggesting that periostin may be significant in controlling tumor progression towards metastasis (Bao et al., 2004; Ruan et al., 2009).

Finally, periostin has a role as a mediator of inflammation through its interactions with inflammatory cytokines (Cobo et al., 2016). Chronic inflammation of the airway is one of the hallmark features of asthma. Woodruff *et al.* (2009) demonstrated that the T-helper type 2 (Th2) cells are at least partially responsible for propagating the inflammatory response in asthmatics (Woodruff et al., 2007). Specifically, Th2 cells produce the inflammatory cytokine, interleukin (IL)-13, which induces expression of periostin by bronchial epithelial cells leading to airway hyper-responsiveness, inflammation, mucous production and activation and proliferation of airway fibroblasts (Woodruff et al., 2007). Additionally, periostin can act as a guide to facilitate granulocyte infiltration, which supports the inflammatory response (Johansson, Annis, & Mosher, 2013). The inflammatory response is active during the allergic response when periostin expression is up-regulated by type-2 inflammatory cytokines (G. Takayama et al., 2006; Woodruff et al., 2007, 2009). A literature review by Izuhara *et al.* (2014) defined periostin's role in allergic inflammation as a downstream effector of the inflammatory cytokines, IL-4 and IL-13

(Izuhara et al., 2014). Reduction in periostin expression or blockage of the periostin-integrin interaction has been demonstrated to reduce the intensity of this Th2-mediated inflammatory allergic reaction, signifying the potential for periostin to be used in a therapeutic manner (Izuhara et al., 2014).

Collectively, these studies suggest that periostin has a role in cell adhesion that facilitates cellular growth under normal physiological conditions. However, abnormal periostin control has been implicated in pathological conditions such as injury wound healing, metastatic growth and inflammation as a consequence of defects in cell adhesion and connective tissue integrity. The specific roles of periostin on a molecular level are still vague and unclear, but what is certain is the fact that periostin's influence touches all facets of normal development and physiology.

Despite the widespread expression of periostin across many tissues, the most abundant levels of periostin by far are found in the PDL, the tissue of most interest to the dental field (Yamada et al., 2014). Immunohistochemistry studies have revealed the highest levels of periostin expression in the PDL with a primary localization to the fibroblasts of the PDL and osteoblasts of the alveolar bone proper (Horiuchi et al., 1999; I. Takayama & Kudo, 2012; Wilde, Yokozeki, Terai, Kudo, & Moriyama, 2003; Yamada et al., 2014). The PDL consists of the following cell types: fibroblasts, osteoblasts, cementoblasts, osteoclasts, mast cells and undifferentiated periodontal ligament stem cells (PDLSCs) (Matsuzawa et al., 2015; Seo et al., 2004). The PDL is represented as a tissue with rapid turnover, endless remodeling and high regenerative capacity in relation to other connective tissues (Beertsen, 1975; Matsuzawa et al., 2015; Sodek, 1977). Most of these characteristics are attributed to the fibroblasts and PDLSCs, which play important roles in maintaining and supporting the PDL (Matsuzawa et al., 2015; Seo

et al., 2004). As in other connective tissues, periostin is a key component to maintaining the homeostasis of the PDL, which withstands the continuous day-to-day stresses of mastication. Periostin maintains the integrity of the PDL allowing it to adapt to and cushion occlusal forces, while simultaneously relaying sensory feedback to the masticatory system during such functions (Yamada et al., 2014). During oral development, periostin has also been reported to exist within developing tooth buds between the epithelium and mesenchyme, a location that further suggests its role in ECM organization within the oral tissues (Ma et al., 2011). The PDL contains undifferentiated PDLSCs, which are capable of differentiating into mineral forming cells such as osteoblasts and cementoblasts (Seo et al., 2004; Yamada et al., 2014). Not only is periostin correlated with osteoblast differentiation from pre-osteoblasts, the up-regulation of periostin has also been shown to increase the adhesion to and overall cohesiveness of the ECM and limit the total migration of pre-osteoblasts *in vitro*, indicating the impact of periostin on bone cell development and physiology (Cobo et al., 2016). Contrary evidence was found by Matsuzawa *et al.* (2015) as periostin was, in fact, shown to promote the migration of PDLSCs through the integrin  $\alpha\beta3$ -FAK signaling pathway, which illustrates the complex nature of periostin involvement in migratory control (Matsuzawa et al., 2015). Periostin also plays a role in angiogenesis of the PDL as it has been demonstrated to up-regulate matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) through the integrin  $\alpha\beta3$ -ERK signaling pathway; MMP-2 and VEGF are two factors critical for angiogenesis (Watanabe, Yasue, Fujihara, & Tanaka, 2012). Similar findings were recorded by Yamada *et al.* (2014) as they demonstrated that periostin within the PDL had the highest binding affinity towards the integrin  $\alpha\beta3$ , suggesting that cytodifferentiation of osteoblasts, angiogenesis and osteoblast migration all likely occur through this particular integrin (Yamada et al., 2014). Thus, periostin

increases cell migration, recruitment and attachment to the PDL as well as promotes angiogenesis, key processes underlying orthodontic tooth movement (OTM) and periodontal wound healing (Romanos et al., 2014; Watanabe et al., 2012; Yamada et al., 2014). During OTM, periostin expression has been identified to increase on the compressive side and also known to be up-regulated by hypoxia (P. Li, Oparil, Feng, & Chen, 2004; Ouyang et al., 2009; Wilde et al., 2003). By promoting differentiation and migration of fibroblasts and osteoblasts, periostin may regulate OTM by controlling the rate of PDL and alveolar bone turnover as well as regulating angiogenesis (Romanos et al., 2014). Periodontal wound healing is likely also regulated through similar mechanisms of action (Romanos et al., 2014). Within the PDL, periostin maintains homeostasis and enhances OTM and periodontal wound healing through its regulation of ECM integrity, cell migration, cell differentiation and angiogenesis of new blood vessels (Romanos et al., 2014). Periostin's fundamental influence within the PDL suggests that therapeutic control to enhance or suppress periostin expression could be important in orthodontics, periodontics and all aspects of dentistry.

Because of periostin's wide array of functions and influences within the PDL and in other tissues, transcriptional control of periostin is paramount to ensuring the proper degree of expression of the protein. Numerous transcription factors are involved along many complex pathways to regulate the transcription and ultimately, expression of periostin. Factors controlling osteoblast differentiation and subsequent bone mass, bone strength and bone remodeling include: Runx2/cbfa1, Wnt/ $\beta$ -catenin and osterix, all of which play a role in committing pluripotent mesenchymal cells towards the osteoblast lineage (Toshihisa Komori, 2006; B. Merle & Garnero, 2012). In particular, overexpression of Runx2 has been shown to be positively associated with periostin expression, suggesting its role in early osteoblast differentiation (Stock,

Schäfer, Fliegauf, & Otto, 2004). Surprisingly, Wnt-3 was demonstrated to be a negative regulator of periostin, which suggests that not all factors within the Wnt pathway affect periostin expression in the same manner (Haertel-Wiesmann, Liang, Fantl, & Williams, 2000). Twist-1 is another transcription factor with both positive and negative controls on periostin expression. Specifically, Twist-1 homodimers bind to the periostin promoter region and up-regulate its transcription whereas Twist-1 heterodimers do the exact opposite and down-regulate its transcription leading to an increase or decrease in osteoblast differentiation respectively (Connerney et al., 2006; Oshima et al., 2002). Thus, transcriptional control of periostin appears to be dynamic and multi-factorial in nature. Another transcription factor involved in periostin regulation is c-Fos/AP-1. Mice overexpressing c-FOS developed sclerotic lesions in bone with differentiated osteoblasts showing high levels of periostin, which was not evident in normal osteoblasts (Kashima et al., 2009). Similarly in humans, patients with fibrous dysplasia demonstrated osteosclerotic bone lesions with increased expression of c-Fos and high levels of periostin, underscoring the transcriptional control of c-Fos over periostin (Kashima et al., 2009). External influences over periostin expression also exist through various cytokines, hormones and growth factors. Parathyroid hormone (PTH) and sex steroids are anabolic elements, which increase bone mass. PTH treatment *in vitro* has been demonstrated to enhance periostin expression and bone deposition through ERK, BMP and Wnt signaling pathways (Ogita, Rached, Dworakowski, Bilezikian, & Kousteni, 2008); PTH may also enhance bone deposition by inhibiting sclerostin, an inhibitory element on bone formation (Ogita et al., 2008). PTH works to enhance bone formation using multiple avenues of control. Estrogens have also been reported to stimulate osteoblast differentiation and bone formation by increasing alkaline phosphatase and osteocalcin activity (Mamalis, Markopoulou, Lagou, & Vrotsos, 2011). Other studies have

showed more ambiguous results where estrogens increase immature periosteal cell proliferation, but not differentiation by regulating PTH and BMP2, which suggests that periostin is controlled in many ways (Ogita et al., 2008). Other prominent cytokines known to enhance periostin expression are platelet-derived growth factor (PDGF), basic fibroblast growth factors (FGF-1 and FGF-2), angiotensin II and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), although not all have been demonstrated *in vivo* (B. Merle & Garnero, 2012). Environmental conditions such as mechanical stress and hypoxia stimulate periostin expression as a way to increase cell survival under harmful conditions (Ouyang et al., 2009; H. F. Rios et al., 2008). Finally, several members of the TGF- $\beta$  superfamily of proteins have also been demonstrated to stimulate periostin expression in osteoblasts namely, TGF- $\beta$ , BMP2, activin and retinoic acid (Eijken et al., 2007; Horiuchi et al., 1999; G. Li et al., 2006; Lindner et al., 2005; Wen et al., 2010). In summary, periostin affects predominantly pre-osteoblasts rather than mature osteoblasts and can thus be viewed as a marker for pre-osteoblasts. Its regulation is one that is dynamic, complex and significant to modulate the degree of osteoblast differentiation and subsequent bone formation within the PDL (B. Merle & Garnero, 2012).

As the crux of this study focuses on the association between periostin and BMPs, the next section of this literature review will discuss BMPs in depth followed by the rationale for its selection for study.

## 1.2: Bone Morphogenetic Proteins (BMPs)

At the end of the nineteenth century, ground demineralized bone matrix material was already being used as an aid in bone healing in cases of bone fracture. It was not until the 1960s, however, when the significance of BMPs was first noted by Urist (1965) for their osteoinductive

potential in bone formation (Carreira et al., 2014; Marshall R Urist, 1965). This discovery was important as it spurred the beginning of a path towards keying in on the role of this group of proteins in bone differentiation and formation. In 1979, Urist *et al.* (1979) furthered the literature again when they isolated BMPs, characterized them as glycoproteins and demonstrated their innate ability to induce bone morphogenesis, both within and between species (M R Urist, Mikulski, & Lietze, 1979). Their findings suggest that BMPs possess a highly conserved structure that translates to function, which is maintained across the animal kingdom. In the 1980s, Wozney *et al.* (1988) isolated and cloned the first BMPs from bovine serum extract and demonstrated that they could each independently direct bone differentiation and formation (Wozney et al., 1988); these BMPs were BMPs 1, 2 and 3. This discovery solidified the significance of BMPs as a critical component of bone morphogenesis and led to further experiments to fully characterize these proteins. Since then, more than 20 different types of BMPs have been identified in humans and other species, each playing a crucial role in the development and role of the tissue type (Carreira et al., 2014). Surprisingly, despite the nomenclature, BMPs are not just involved in osteogenesis (Lissenberg-Thunnissen, De Gorter, Sier, & Schipper, 2011). BMPs have been noted to be involved in embryogenesis as well as the development of other tissue systems as well. Specifically, BMPs have been shown to regulate the development of the teeth, nervous system, eye, lung, heart, kidney and genitalia (Bragdon et al., 2011; Oryan et al., 2014). To appreciate the extensiveness of their influence, BMPs can be subdivided into four main categories based on sequence similarity and function: BMPs 2 and 4; BMPs 5, 6, 7, 8a and 8b; BMPs 9 and 10; and finally, BMP3. The first three classes have been recognized to be osteogenic, but the last class, BMP3, is a notable inhibitor of osteogenesis (Jain, Pundir, & Sharma, 2013; Lissenberg-Thunnissen et al., 2011; Tsiridis, Upadhyay, & Giannoudis,

2007). Of all of the BMPs, BMPs 2, 4, and 7 show the most promise as they each have intrinsic osteogenic potential (Carreira et al., 2014).

With the exception of BMP1, which is part of the metalloprotease group of proteins, BMPs are considered to be a part of the TGF- $\beta$  superfamily of proteins with conserved structural elements and derived from approximately 50 genes (Carreira et al., 2014). BMPs are dimeric, glycoproteins with sites for N- and O-linked glycosylation, which increase the stability and efficacy of these proteins in the body. The fully transcribed peptide sequence of BMPs consists of a signal peptide at the N-terminus, a polypeptide sequence of the mature protein at the C-terminus and a non-conserved prodomain region connecting the two termini, which controls the proper folding of the protein (Ali & Brazil, 2014). After translation, the premature protein is cleaved by subtilisin-like convertase (SCP) to produce the mature protein, which spans 100-140 amino acid residues in length (Carreira et al., 2014). Its structure contains seven conserved cysteine residues total, six of which form three intramolecular disulfide bonds known as cysteine knots. The remaining cysteine residue is involved in forming an intermolecular disulfide bond with another BMP monomer producing the final, biologically active BMP homodimer; BMPs only exist as homodimers in the body as this relationship is both critical and necessary to their biological activity (Carreira et al., 2014). In essence, BMP homodimers are the predominant form of signaling of each type of BMP and are antagonized by other homodimeric proteins such as noggin and gremlin (W. Zhu et al., 2006).

The mechanism of action by which BMPs elicit their effects is a tightly controlled, complex signaling cascade with regulators at every level. The process begins with the binding of BMPs to one of two types of transmembrane serine/threonine kinase receptors: type I (BMPR-1)



and type II (BMPR-II) kinase receptors (Rosenzweig et al., 1995). Within each type of receptor, there are three subcategories of receptors, which BMPs preferentially bind. For BMPR-I, the subtypes are: activin receptor-like kinase (ALK) 2, ALK3 (BMPRIA) and ALK6 (BMPRIB). As for BMPR-II, the subtypes include: BMP type II receptor (BMPR2), activin A receptor type II (ActR2) and activin A receptor type IIB (ActR2B) (Nohe, Keating, Knaus, & Petersen, 2004). Within the type I category, the binding affinity for each receptor subtype varies depending on the BMP. For instance, BMP4 has a higher affinity towards ALK3 and ALK6 whereas for BMPs 6 and 7, ALK2 is the preferred receptor (Aoki et al., 2001). In most cases, BMPs initially either bind to type I receptor or preformed type I/type II receptor complexes. If bound to a type I receptor first, this ligand-receptor complex then recruits a constitutively active type II receptor, which catalyzes the trans-phosphorylation of the type I receptor at its glycine and serine rich cytoplasmic domain (GS domain), thereby activating the type I receptor (Miyazono, Kamiya, & Morikawa, 2010). This conduit is not the only method of activation, however. There are some BMPs such as BMP7, which bind first to the type II receptor followed by type I receptor recruitment and subsequent phosphorylation (Oryan et al., 2014). Though, it is believed that BMPR-II does not actually bind to the ligand, but instead, mediates the interaction between the ligand and BMPR-I or merely accelerates this phenomenon (Oryan et al., 2014). Nevertheless, the activated BMPR-I proceeds to phosphorylate downstream Smads1, 5 and 8, which are primarily responsible for controlling gene expression (Oryan et al., 2014).

In the realm of cell signaling, Smad proteins play a crucial role in propagating and mediating the downstream signal, ultimately modifying gene expression within the nucleus. Smad proteins are homologs of both the *Drosophila melanogaster* mothers against decapetaplegic and related, *Caenorhabditis elegans* Sma gene (Ali & Brazil, 2014; Riggins et al.,

1996). These proteins are intracellular mediators that transduce extracellular signals from the TGF- $\beta$  superfamily of proteins, such as BMPs, to the cell nucleus where they modify gene transcription of targeted genes (Ali & Brazil, 2014). Currently, there are a total of eight known Smads that are divided into three groups based on their functions. Smads1, 2, 3, 5 and 8 are known as receptor Smads (R-Smads). Among the R-Smads, Smads1, 5 and 8 are the substrates for BMP receptors and upon activation, act downstream to enable transcription of targeted genes (Oryan et al., 2014). The second group contains the common mediator Smad (co-Smad), Smad4, which works in concert with R-Smads to facilitate their effects (Oryan et al., 2014). Finally, the last group is the inhibitory Smads, Smad6 and Smad7, which antagonize the effects of R-Smads and prevent their association with the co-Smad (Oryan et al., 2014). After activation and formation of the ligand-receptor complex at the cell surface, cytoplasmic R-Smads associate with the co-Smad mediator, Smad4, forming a Smad/co-Smad complex (Ali & Brazil, 2014). This complex is the active constituent responsible for regulating gene expression and thus, its formation is absolutely critical to fully realize the effects of BMPs. Together, this protein complex translocates to the nucleus to modify gene expression of key transcription factors responsible for bone and cartilage formation: Runx2, Dlx5, Osterix and Sox2 (Ali & Brazil, 2014; Carreira et al., 2014; W. Shi et al., 2007). The significance of these transcription factors is seen in cases of Runx2 mutations. Specifically, patients with Runx2 mutations develop cleidocranial dysplasia exhibiting hallmark features such as short stature and the lack of a clavicle (F Otto, Kanegane, & Mundlos, 2002). Runx2 deficient mice are also completely nonviable due to severe faults in osteogenesis, highlighting the importance of Runx2 and other transcription factors along the Smad signaling pathway for osteoblast differentiation (T. Komori et al., 1997; Florian Otto et al., 1997). Deletion of Runx2 and Osterix inevitably results in the

loss of bone formation (Carreira et al., 2014). There are, however, antagonistic elements to the Smad pathway, which act as a layer of control. Extracellular antagonists include noggin, chordin, twisted gastrulation (Tsg), gremlin and follistatin, all of which contain cysteine-knots which attract and bind to BMPs, effectively preventing their association with their respective receptors (Carreira et al., 2014). There are intracellular inhibitors as well: Smad6, Smad7, Smad8b, Smurf1 and Smurf2 (Carreira et al., 2014; C. Suzuki et al., 2002). These intracellular antagonists either interfere with the Smad/co-Smad association or direct the Smad/co-Smad complex towards proteosomal degradation. In both cases, the Smad/co-Smad complex is eliminated entirely, preventing signal propagation and the modification of gene expression affecting bone and cartilage development (Carreira et al., 2014). The Smad pathway is the principle pathway by which BMPs elicit their effects and the numerous regulators helps to ensure that the proper downstream response is achieved.

While the Smad pathway is the predominant form of signaling for BMPs, there are also Smad-independent or non-Smad pathways of signal transmission via mitogen activated protein kinases (MAPK), small Rho GTPase and Akt pathways (Oryan et al., 2014). As the nomenclature suggests, MAPKs work without the use of Smads, but rather utilize other protein kinases instead for signaling. The beginning of Smad-independent pathways operate in a similar fashion to Smad-dependent signaling with BMPs initially binding to a type I receptor, which leads to the recruitment of an active type II receptor for trans-phosphorylation (Oryan et al., 2014). BMPs can also bind to preformed complexes of type I/type II receptors to trigger the downstream signaling cascade. Upon phosphorylation, BMPR-I becomes active and transduces the signal to downstream kinases in an interaction catalyzed by a three membered protein complex consisting of: TAK1 (MAP3K7IP1), co-activator TAB1 (MAP3K7) and X-linked

inhibitor of apoptosis protein (XIAP) (Oryan et al., 2014). Altogether, this protein complex works in tandem with the activated BMPR-I to facilitate phosphorylation of downstream MAPKs in a role similar to Smad4 in Smad-dependent signaling. The targeted MAPKs in this pathway include: p38 (MAPK14), ERK (MAPK1) and JNK (MAPK8) (Oryan et al., 2014). Osteoblast differentiation and subsequent bone formation are reliant upon the p38/ERK MAPK pathway (Oryan et al., 2014). The ERK MAPK and TAK1 proteins also have important roles in regulating BMP Smad-dependent signaling, which suggests that the two BMP signaling pathways may not function independently of one another, but rather in a cooperative manner. TAK1 possesses a dual role and functions as both a BMP agonist and antagonist. Specifically, TAK1 synergizes with Smads 1 and 5 as an agonist, but also can behave as an inhibitor by binding and interfering with R-Smad trans-activation, ultimately inhibiting BMP-induced osteodifferentiation (Beederman et al., 2013; Oryan et al., 2014). These contrasting functions suggest that TAK1 may be a key modulator in BMP response. TAK1 is also able to independently promote phosphorylation of Smads1, 5 and 8 and thus, regulate control of the Smad pathway on its own, further underscoring the importance of this protein in BMP regulation along both Smad-independent and Smad-dependent pathways (Oryan et al., 2014; S. Shi, de Gorter, Hoogaars, 't Hoen, & ten Dijke, 2013). Currently, it is unclear how the versatility and functionality of TAK1 is regulated and able to act upon both Smad-independent and dependent pathways. Upon phosphorylation, MAPKs translocate to the nucleus whereby they activate the above mentioned osteogenic transcription factors initiating gene expression (Oryan et al., 2014). In addition to MAPKs, Smad-independent signaling can proceed along other pathways as well namely, small Rho GTPase and Akt pathways (Oryan et al., 2014). Activation of PI3K, a non-MAPK member, results in signal transduction along these pathways that affect gene transcription (Oryan et al.,

2014). The complexity and overlapping functions of the various proteins within each pathway suggests that BMP signal propagation is not a simple process, but instead one that is deeply intertwined and not solely governed by any single pathway or regulator.

Outside of Smad and non-Smad regulation, there are, in fact, additional mechanisms of control in BMP signaling. As Smad-independent and Smad-dependent regulation are arguably the primary players in this process, the following controllers can be considered auxiliary regulators in BMP signaling (Ali & Brazil, 2014; Oryan et al., 2014). CRIM1 is a transmembrane protein containing six cysteine-rich repeats that regulates the delivery of BMPs to the cell surface (Carreira et al., 2014). The cysteine-rich domains of CRIM1 have a strong binding affinity towards the cysteine knots of the BMPs. As a result of this interaction, CRIM1 limits the amount of mature BMP that is secreted, thereby controlling the degree of osteogenic gene activation of the target cell (Wilkinson et al., 2003). Another transmembrane protein, BAMBI pseudo-receptor (BMP and activin membrane-bound inhibitor), possesses sequence similarity to type I receptors which allows it to block BMP signaling via competitive inhibition and thereby prevent the formation of the ligand-receptor complex necessary to propagate the signal to downstream kinases (Onichtchouk et al., 1999). Endoglin (CD105) is another transmembrane protein involved in inhibiting the TGF- $\beta$  and BMP signaling pathway (Ishibashi et al., 2010). Protein phosphorylation is a common cellular mechanism of activation and how kinases function as regulators. Not surprisingly, protein dephosphorylation plays a vital role in the regulation of BMP signaling as well. Proteins involved in dephosphorylation are called phosphatases and function by inactivating targeted proteins through the removal of a phosphate group (Ali & Brazil, 2014). Specifically, protein phosphatase-1 (PP1) dephosphorylates BMP receptors whereas protein phosphatase 1A (PPM1A) dephosphorylates R-Smads, both of which

inhibit BMP signaling in their own right through the removal of phosphate groups (Ali & Brazil, 2014; Lin et al., 2006; Weibin Shi et al., 2004). From extracellular to intracellular control, it is clear that the BMPs are tightly regulated on multiple levels through auxiliary regulators to ensure that the appropriate cellular response is achieved and maintained.

Despite the number of BMPs discovered, only a select few have been shown to be involved in osteogenesis. The remainder of this review on BMPs will focus on those BMPs having a role in bone formation and bone differentiation. The human skeleton consists primarily of cartilage and bone that is under a constant state of remodeling by chondrocytes, osteoblasts and osteoclasts respectively (Ali & Brazil, 2014). Chondrocytes are responsible for maintaining the integrity and metabolic balance of the cartilage whereas osteoblasts and osteoclasts are the bone forming and bone resorbing cells within the bone respectively (Tanaka, Nakayamada, & Okada, 2005). As previously discussed, bone remodeling overseen by these cells is a tightly controlled process involving BMPs and the downstream signaling cascade of R-Smads. Secreted BMPs affect bone remodeling in one of three ways: 1) bind and activate BMP receptors to initiate the signaling cascade in a stimulatory fashion, 2) become inhibited by secreted antagonists that suppress BMP action or 3) bind ECM proteins such as collagen and act as a reservoir of BMP for future use (Ali & Brazil, 2014; Miyazono et al., 2010). BMP2, BMP4 and most recently, BMP7 are three primary BMP candidate proteins that have been shown to be involved in initiating bone formation and maintaining bone homeostasis (Carreira et al., 2014; Deschaseaux, Sensébé, & Heymann, 2009). In one study, BMP2 was injected onto the calvaria of mice resulting in bona fide periosteal bone formation on the surfaces of the calvaria (D. Chen et al., 1997). Another study demonstrated the ability of BMP2 to restore mineralization in inhibited osteoblast cultures, highlighting the importance of BMP2 as an osteogenic factor and a

potential initiator in the bone formation process (Luppen, Smith, Spevak, Boskey, & Frenkel, 2003). Together, BMP2 and BMP4 were also shown to be powerful inducers of chondrocyte and osteoblast differentiation, key processes leading to cartilage and bone development respectively (Nishimura, Hata, Matsubara, Wakabayashi, & Yoneda, 2012). BMP2 and BMP4 are so critical to this process that their combined loss led to a severe failure in osteoblast differentiation altogether (Oryan et al., 2014; Stewart, Gomez, Armstrong, Henner, & Stankunas, 2014). The significance of these proteins is so deeply intertwined with skeletogenesis and growth and development that BMP2 and BMP4 knockout mice are completely nonviable. In two separate studies, homozygous knockout BMP2 and BMP4 mice died shortly after birth and showed severe developmental abnormalities related to the heart, skeleton and mesoderm (Winnier, Blessing, Labosky, & Hogan, 1995; Zhang & Bradley, 1996). Although less thoroughly examined, BMP7 has also been shown to have osteogenic activity. In one study, various cells were induced to differentiate into osteoblasts upon BMP7 gene transduction using an adenovirus vector (Franceschi, Wang, Krebsbach, & Rutherford, 2000). In another instance, both BMP2 and BMP7 genes were transduced in a rodent model and resulted in significantly increased osteoblast activity more so than any one individual BMP gene transfer alone. Not only is BMP7 a critical factor in regulating osteoblast differentiation, it may also act in a synergistic manner with other osteogenic BMPs to further accelerate osteogenesis as suggested by this study (W. Zhu et al., 2004). A more recent study demonstrated that human recombinant BMP7 stimulates osteodifferentiation and *in vivo* bone formation, further underlining the significance of BMP7 and marking a shift towards clinical applicability of BMPs (F. Chen et al., 2017). There is one notable BMP that is antagonistic to osteodifferentiation and it is BMP3. BMP3 has been shown to inhibit osteogenesis by antagonizing the effects of BMP2 and BMP4 (Daluisi et al., 2001;

Gamer, Nove, Levin, & Rosen, 2005). BMP3 also plays a role in fracture healing as well as modulating the effects of osteogenic BMPs to maintain bone homeostasis (Chang, Lu, Shibata, Tsukazaki, & Yamaguchi Dr., 2012; Oryan et al., 2014). Overexpression of BMP3 in transgenic mice, however, induced spontaneous fractures, suggesting that BMP3 expression and its influence on the bone remodeling process is tightly controlled (Gamer, Cox, Carlo, & Rosen, 2009). The most recent evidence indicate that BMP3 has an overwhelming potential to suppress osteodifferentiation in progenitor bone cells more than initially believed in the past. The ability of BMP3 to reverse cells predetermined to become osteoblasts illustrates the penetrating inhibitory ability of BMP3 in the bone remodeling process and underscores the importance of this protein as an osteogenic modulator (Kokabu et al., 2012). Although most of the studies have been performed on mice, previous studies on BMPs 2, 3, 4 and 7 collectively illustrate the dynamic interplay between agonistic and antagonistic elements to moderate the bone remodeling process, an interaction which likely carries over to some extent in humans as well.

The findings of BMPs as the underlying mechanism in the bone remodeling process is pioneering, but also raises the question as to whether there is any clinical applicability to BMPs and if so, how to best employ them pharmacologically to treat disease. In order to utilize BMPs as pharmaceuticals in humans, the cDNAs of the BMPs are first cloned and reproduced using genetic recombination techniques to create biologically active, human recombinant BMPs (rhBMPs) (Carreira et al., 2014). RhBMPs can then be strategically applied at target sites to enhance bone remodeling. The only two BMPs to achieve full FDA approval for use in patient care are BMPs 2 and 7 in their recombinant forms as rhBMP2 and rhBMP7 respectively (Carreira et al., 2014). Between the two rhBMPs, rhBMP2 is the most widely studied and utilized rhBMP for its therapeutic benefits in orthopedics and in dentistry (Ali & Brazil, 2014).



Studies have demonstrated clinical efficacy of rhBMP2 in cases of open or closed long bone fractures, maxillofacial defects, joint arthrodesis and in particular, spinal fusion of various types (Aro et al., 2011; Fourman, Borst, Bogner, Rozbruch, & Fragomen, 2014; Lyon et al., 2013; Marx, Armentano, Olavarria, & Samaniego, 2013; Oryan et al., 2014; Roh, Yeung, Field, & McClellan, 2013). In one study, patients with open tibia fractures were treated with standard surgical fixation to facilitate fracture healing, which was supplemented with either rhBMP2 or a placebo. The rhBMP2 and placebo were delivered to the site using an absorbable type I collagen sponge to enhance uptake of the materials. The results of this study showed that patients treated with rhBMP2 had accelerated fracture and wound healing, in addition to less surgical complications such as infection, compared to the placebo group (Ali & Brazil, 2014; Govender et al., 2002). Follow-up studies have also proved the clinical efficacy of rhBMP2 treatment under similar scenarios as well as in combination with bone allografting (Nauth, Ristiniemi, McKee, & Schemitsch, 2009; Swiontkowski et al., 2006). Although not as extensively studied as rhBMP2, rhBMP7 has also been shown to accelerate wound healing and reduce the need for secondary procedures when used together with surgical repair of fractures (Ristiniemi et al., 2007). RhBMP7 may also be efficacious in controlling osteoarthritis by inducing ECM collagen formation to counteract the breakdown of articular cartilage in the joints (Fan et al., 2004). In dentistry, rhBMPs have been implicated for off-label usage in periodontal regeneration, bone healing, implant osteointegration, ridge augmentation and oral surgery (Carreira et al., 2014; Hong, Boyd, Beyea, & Bezuhy, 2013). With both rhBMP2 and rhBMP7, collagen sponge delivery media were historically used, but recent studies have experimented with hybrid nanofiber mesh/alginate media, among others, to further enhance rhBMP uptake (Boerckel et al., 2011). Currently, novel approaches utilizing mesenchymal stem cells as the transport medium for

rhBMPs are being tested (Liebergall et al., 2013). Despite the therapeutic potential of rhBMPs, numerous undesirable side effects have been noted including hematoma, swelling, surgical site infection, wound complication, ectopic bone formation and bone resorption (Ali & Brazil, 2014; Oryan et al., 2014). It has been suggested that some of these side effects may be from the inductive nature of rhBMPs on the inflammatory host response (Arrabal, Visser, Santos-Ruiz, Becerra, & Cifuentes, 2013; Oryan et al., 2014). Together with the high treatment cost of rhBMPs, further studies are necessary to outline the best indications for their usage so that clinical efficiency and efficacy are maximized while side effects and costs are limited (Ali & Brazil, 2014; Garrison et al., 2010).

### 1.3: Periostin & Bone Morphogenetic Proteins

The relationship between periostin and BMPs was first explored by Horiuchi *et al.* (1999) during periostin's discovery (Horiuchi et al., 1999). It was shown that TGF- $\beta$ 1 and BMP2, members of a greater TGF- $\beta$  superfamily of proteins, increase the expression of periostin to initiate bone formation (Horiuchi et al., 1999). Delving into this relationship further showed that TGF- $\beta$ 1 increases periostin expression only up until the point that bone formation occurs, at which point periostin expression actually decreases, suggesting that TGF- $\beta$ 1 may have a role in regulating the beginning of bone mineralization (Eijken et al., 2007). The association between periostin and BMPs garnered scientific interest upon its inception by Horiuchi *et al.* (1999) and the interest was maintained as more studies further validated this connection (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). Ji *et al.* (2000) examined the association between another member of the TGF- $\beta$  superfamily namely, BMP2, and periostin, and demonstrated that it too increased the expression of periostin (Ji et al., 2000). These findings

collectively showed that members of the TGF- $\beta$  superfamily of proteins could indeed regulate periostin expression and perhaps even manipulate its expression to achieve a desired response (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). The versatility of BMPs in clinical applications in orthopedics, dentistry and other disciplines begs the question as to whether BMPs can effectively and predictably affect periostin expression (Horiuchi et al., 1999; Hussein et al., 2013; Papakostidis, Kontakis, Bhandari, & Giannoudis, 2008). As discussed earlier, BMPs are tightly controlled cytokines with a wide spectrum of roles ranging from development to bone and cartilage formation and repair (Oryan et al., 2014). As pharmaceuticals, they have also been effective primarily as adjuncts to accelerate wound healing in medicine and in dentistry, further validating their significance (Oryan et al., 2014). The overlapping roles of periostin and BMPs in osteoblast differentiation and wound healing present an interesting opportunity to determine the significance of this relationship and how it can be controlled and applied therapeutically. As a symbolic marker of osteoblasts, periostin expression can be interpreted as a sign of sustained osteoblast differentiation and proliferation, the control of which could be potentially significant under various clinical settings. Although TGF- $\beta$ 1 and to a lesser extent, BMP2, have been examined with respect to periostin expression in bone cells, there have not been any reports on the effects of other BMP family members on periostin expression, despite a shared homology between the BMPs. Given that different BMPs are differentially expressed in tissues of the body with different physiological functions, it is reasonable to assume that they may have different effects on periostin expression (Oryan et al., 2014). Other than BMP2, only a select few BMPs are known modulators of osteogenesis and they include: BMP3, BMP4 and BMP7. Previous experiments have demonstrated the osteogenic potential of BMP4 and BMP7 and thus, they are expected to have similar effects on periostin expression (Franceschi

et al., 2000; Nishimura et al., 2012; S. Zhu et al., 2009). BMP3, however, has previously been demonstrated to have a modulation type of effect and could enhance or suppress periostin expression (Chang et al., 2012; Daluiski et al., 2001; Gamer et al., 2005). To date, the effect of varying concentrations of BMPs has not been examined. By analyzing the effect of BMP concentration on periostin expression, a more global understanding of the dynamics of this relationship may be conferred.

#### 1.4: Research Questions

Based on the periostin and BMP literature review, research questions were formulated to guide the investigation and focus the experiments accordingly. The following research questions were asked and null and alternative hypotheses presented as follows:

- i. Do BMP2, BMP4 and BMP7 treatment increase periostin expression in pre-osteoblast cells?

$H_0$  – BMP2, BMP4, BMP7 do not increase periostin expression.

$H_A$  – BMP2, BMP4, BMP7 increase periostin expression.

- ii. Does BMP3 treatment reduce periostin expression in pre-osteoblast cells?

$H_0$  – BMP3 does not decrease periostin expression.

$H_A$  – BMP3 does decrease periostin expression.

- iii. For all BMPs, do increasing concentrations cause significant changes in periostin expression in pre-osteoblast cells?

$H_0$  – Concentration does not affect periostin expression.

$H_A$  – Concentration does affect periostin expression.

## 1.5: Study Objective

The objective of this study was to demonstrate the *in vitro* expression of periostin in MC3T3-E1 mouse pre-osteoblast cells in response to different BMPs at varying concentrations and if present, to quantify the differences in periostin expression. MC3T3-E1 cells were selected as they are the gold standard murine cell for studying pre-osteoblast cells and have also been employed in previous studies on periostin. Previous studies describing the regulation of periostin expression by TGF- $\beta$ 1 suggests that periostin has the potential to be a downstream effector to treatment by the TGF- $\beta$  superfamily of proteins (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). In this study, the expression of periostin was hypothesized to increase with BMP2, BMP4 and BMP7 treatment, supporting the notion of these BMPs as enhancers or agonists of periostin expression. In contrast, BMP3 was hypothesized to suppress periostin expression due to its innate inhibitory potential. However, BMP3 does possess modulator activity and could also enhance periostin expression under the right conditions as well. For all BMPs examined in this study, varying concentrations were hypothesized to have an effect on periostin expression. To be specific, periostin expression was expected to increase, as in the case for BMP2, BMP4 and BMP7, and decrease, as in the case for BMP3, with higher concentrations of the respective BMPs. Unlike other studies, this research was unique in attempting to determine not only the effects of BMP2, but also BMP4 and BMP7 on periostin expression, which, to our knowledge, have never been considered. In addition, no previous studies have considered the modulatory effects of BMP3 on periostin expression. The effect of varying BMP concentrations adds another layer of complexity to this study as well. By incorporating both hypothesized agonists (BMP2, BMP4 and BMP7) and antagonists (BMP3) to periostin expression in the study, a more complete understanding of periostin regulation can be achieved.

Ultimately, understanding the effects of BMPs on periostin expression will contribute to our overall understanding of the complex mechanisms involved in osteoblast differentiation and proliferation as well as their therapeutic applications in the clinical setting. This knowledge may have important clinical implications in the modulation of osteoblast activity, which may be applicable to the dental field in the regulation of tooth movement, regeneration of the periodontium and even *de novo* bone formation.

## Chapter 2: Methodology

### 2.1: Cell Cultivation

MC3T3-E1 mouse pre-osteoblast cells (MC3T3-E1 Subclone 4, ATCC CRL-2593) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Alpha Minimum Essential Medium ( $\alpha$ -MEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone Bovine Growth Serum Supplemented Calf, Logan, UT), 1% penicillin and 1% streptomycin. The  $\alpha$ -MEM also contained 2 mM L-glutamine, ribonucleosides and deoxyribonucleosides. Cells were grown in standard cell culture conditions of 37°C, 95% humidity and 5% CO<sub>2</sub>. Media was changed every 2 days. All plates were examined using light microscopy to confirm adequate confluency (70-80%) before beginning BMP treatment.

### 2.2: BMP Treatment

BMP2 (catalog # 4577-10), BMP3 (catalog # 4573-10), BMP4 (catalog # 4578-10) and BMP7 (catalog # 4579-10) were obtained from BioVision (Milpitas, CA). Each vial containing 10  $\mu$ g of lyophilized BMP was reconstituted to 200 ng/ $\mu$ L in water containing 0.5% BSA and stored at -20°C. A further dilution to 5 ng/ $\mu$ L in water was prepared as a working stock for use in the following treatments: 10 ng/mL (low concentration) and 25 ng/mL (high concentration). All 12-well plates were seeded with cells to provide duplicate wells according to the nine experimental conditions described in Table I. The control group received the same media used to culture cells, with no added BMPs. Treatment groups received specific BMPs in either low (10 ng/mL) or high (25 ng/mL) concentrations depending on the treatment assigned. Two concentrations for each BMP were used to determine concentration-dependent effects on

periostin expression and were selected based on concentrations used in comparable experiments within the literature (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). In all cases, a total of 1 mL of treatment media was used in all groups. Following the addition of BMP-media or media alone, plates containing cells were incubated in an incubator for 24 hours at 37°C, 95% humidity and 5% CO<sub>2</sub>.

Group	BMP Level	BMP Concentration (ng/mL)
Control	N/A	0
BMP2	Low	10
BMP2	High	25
BMP3	Low	10
BMP3	High	25
BMP4	Low	10
BMP4	High	25
BMP7	Low	10
BMP7	High	25

Table I. BMP treatment consisted of nine experimental conditions. Each BMP was used in a low (10 ng/mL) and a high (25 ng/mL) concentration. A control group with no BMP was utilized as a baseline for periostin expression, which all other BMP treatment groups were compared. Treatments were in duplicate and performed in three independent experiments.



### 2.3: RNA Isolation

After 24 hours of incubation, cells were removed from the incubator and visualized under a light microscope to confirm that no significant cell death, aberrant cell growth or unwanted contamination had occurred. RNA isolation was performed using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol to ensure maximum yield. Treatment media was removed from all cell plates using a suction pipettor. TRIzol reagent (600  $\mu$ L) was added to each well containing cells and pipetted vigorously to fully lyse cells and then transferred to Eppendorf tubes to begin RNA isolation. Chloroform (120  $\mu$ L) was added to each tube and pipetted vigorously followed by placement of the tubes on ice to preserve the integrity of the extracted RNA. Eppendorf tubes were spun in a centrifuge (Eppendorf Centrifuge 5415D, Hamburg, Germany) at 11,000 RPM, 4°C for 15 minutes. The aqueous portion (supernatant) containing the RNA was subsequently transferred to a second set of Eppendorf tubes for further purification. 350  $\mu$ L of isopropanol was added to the second set of tubes containing the aqueous portion for RNA separation. The tubes were centrifuged at 12,000 RPM, 4°C for 15 minutes to pellet the RNA. The supernatant was then removed leaving behind a visible, white RNA pellet at the base of the tube. The pellet was re-suspended in 600  $\mu$ L of 75% ethanol and centrifuged again at 10000 RPM, 4°C for 10 minutes as a washing step. Supernatant was removed gently without disturbing the pellet and the RNA pellet was allowed to air dry for 10 minutes before being re-suspended in 25  $\mu$ L of de-ionized, ultra-filtered (DIUF) water. All BMP treatment and RNA isolation experiments were performed in triplicate. Isolated RNA samples were collected and stored at -80°C inside a designated laboratory freezer until all experiments were complete and ready for further processing.

## 2.4: Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Verso 1-step RT-PCR Hot-Start kit (Thermo Fisher Scientific, Waltham, MA) to first reverse transcribe messenger RNA (mRNA) into complementary DNA (cDNA) followed by PCR amplification using specific primers. Before performing RT-PCR on treatment samples, it was important to determine the most ideal primers for this component of the study to maximize the amount of PCR product yield. The two primer sets needed were for mouse periostin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The GAPDH housekeeping gene was selected as a control for cell number to normalize periostin gene expression between the individual experiments. Two sets of periostin (Periostin A and Periostin B) primers and GAPDH (GAPDH A and GAPDH B) primers were identified from previous studies and used with selected samples to determine the primer sets giving the cleanest and greatest yield of amplified product. The primers and their respective sequences are listed in Table II. Each RT-PCR reaction contained a final volume of 25  $\mu$ L and was prepared and run according to the manufacturer's protocol as indicated in Table III.

Primer Set	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
Periostin A*	AACCAAGGACCTGAAACACG	TGTGTCAGGACACGGTCAAT
Periostin B	TGCCCAGCAGTTTTGCCCAT	CGTTGCTCTCCAAACCTCTA
GAPDH A	GCATCTCCCTCACAATTTCCA	GTGCAGCGAACTTTATTGATGG
GAPDH B*	TGCACCACCAACTGCTTA	GGATGCAGGGATGATGTTC

Table II. Periostin and GAPDH primer sets. Two sets of periostin and GAPDH primers were selected to determine the primer set giving rise to the highest yield of PCR product. Sequences for the periostin and GAPDH primers are listed. Periostin A and GAPDH B sets produced the highest yield and were selected for use on all samples in the study (marked with an \*).

Step	Temperature (°C)	Time	Cycles
1) cDNA Synthesis	50	30 min	1
2) Verso Enzyme Inactivation	95	15 min	1
3) Denaturation	95	20 sec	35 total
4) Annealing	55	30 sec	35 total
5) Extension	72	1 min	35 total
6) Final Extension	72	5 min	1

Table III. RT-PCR reaction steps. RT-PCR was performed using a 1-step technique to first reverse transcribe all mRNA into cDNA using random primers. Next, PCR amplification of specific sequences of periostin and GAPDH cDNA was performed until the endpoint was reached. Thermocycling conditions followed the manufacturer's suggested protocol to ensure the highest yield possible.

The RT-PCR reaction was performed with the Verso 1-step RT-PCR Hot-Start kit following the manufacturer's recommendations. The Verso Enzyme Mix included the Verso Reverse Transcriptase, which is responsible for converting mRNA into cDNA. The Mix also included an RNase inhibitor to prevent RNA degradation from occurring as a result of contaminants. The 2X 1-Step PCR Hot-Start Master Mix contained a specialized buffer solution to optimize reverse transcription and PCR amplification in the same reaction vessel. The Master Mix also contained the enzyme for the PCR amplification reaction to catalyze the extension of the cDNA segments: Thermo Scientific Thermo-Start DNA polymerase. RT Enhancer was also included to breakdown any residual DNA and remove contaminants. The thermocycling was performed with an Eppendorf Mastercycler Gradient 5331 (Eppendorf, Hamburg, Germany). In preparation for agarose gel electrophoresis, amplified PCR products (8  $\mu$ L) were combined with 2  $\mu$ L of Thermo Scientific 6X Orange DNA Loading Dye (Thermo Fisher Scientific, Waltham, MA). Samples were loaded and electrophoresed through a 2% agarose gel at 70 volts for 2 hours in a buffer solution of 1X Tris/Borate/EDTA (TBE). One lane of each gel included a DNA standard ladder (Thermo Scientific O'GeneRuler Ultra Low Range DNA Ladder, Waltham, MA) with known base pair (bp) values to assist with confirmation of the corresponding periostin and GAPDH bands. The base pair values for the DNA standard ladder in descending order were: 300 bp, 200 bp, 150 bp, 100 bp, 75bp, 50bp, 35 bp, 25 bp, 20 bp, 15 bp and 10 bp. For both periostin and GAPDH, the bp range of the PCR amplicons were from 171-196 bp (Lee, Lee, Park, & Kim, 2017; Matsuzawa et al., 2015; Tilman et al., 2007). Visualization of gels under UV light showed the most abundant product arising from periostin "A" and GAPDH "B" primer sets. The periostin "A" and GAPDH "B" primers were then used for the RT-PCR of all samples. The selected periostin and GAPDH primers will henceforth be known as merely *periostin* and

*GAPDH* primers with no letter designation, indicating the usage of the most effective primer pairs.

For all samples of the three separate experiments, RT-PCR was performed using the selected periostin and *GAPDH* primers according to the protocol described in Table III. Following RT-PCR amplification, PCR products together with a DNA standard ladder, were loaded onto a 2% agarose gel following the same protocol and settings described earlier. Two gels were run for each of the three sets of experiments: one gel for periostin PCR products and another gel for its corresponding *GAPDH* PCR products. Between the three experimental setups, a total of six gels were run and produced.

## 2.5: Gel Visualization and Analysis

Visualization of the gels was performed under UV light and images of the gels were captured using a specialized Kodak Gel Logic 100 Imaging System (Rochester, NY) linked to its corresponding software, Kodak 1D Image Analysis Software (Rochester, NY). Bands indicative of periostin and *GAPDH* were identified and noted based on the relative base pair values of the PCR products. Gel images were analyzed using the Kodak 1D Image Analysis Software to quantify the pixel density of the different bands, which served as an indication of periostin mRNA expression in each treatment group. For the comparison between experiments, periostin bands were normalized against corresponding *GAPDH* bands for each experiment. After normalization, pixel densities between treatment groups were compared with each other to determine the effect of different BMPs on periostin expression.

## 2.6: Statistical Analysis

All experiments were performed in triplicate. Pixel densities of bands were expressed as normalized values with standard errors and later aggregated to form normalized means and standard error of the means. Changes in band density for each of the BMP treatment groups were compared to the control group individually using the student's t-test with a p-value  $\leq 0.05$ .

## Chapter 3: Results

### 3.1: Periostin and GAPDH Primer Selection

Before starting experiments to assess the relationship between periostin and BMPs, it was first important to identify the most ideal periostin and GAPDH primers to be used during the RT-PCR portion of the experiments in order to maximize the endpoint yield of PCR product.

Although there are numerous periostin and GAPDH primers documented in the literature, it is reasonable to assume that the efficacies of the various primers in PCR amplification may be different. In the first part of this study, we examined two sets of periostin and GAPDH primers gathered from previous studies and tested their effectiveness in RT-PCR on a randomized set of samples (Lee et al., 2017; Matsuzawa et al., 2015; Blandine Merle et al., 2014; Tilman et al., 2007).

Following BMP treatment, cells were lysed and RNA isolation and purification were performed to collect the total RNA within the cells in preparation for RT-PCR. The first step of RT-PCR involved reverse transcriptase, which converted all of the mRNA into cDNA using a variety of non-specific primers. Once all of the mRNA was converted into cDNA, specific primers to periostin and GAPDH were used to propagate the desired amplicons for the periostin and GAPDH genes. Two sets of periostin and GAPDH primers were selected and arbitrarily designated as Periostin A, Periostin B, GAPDH A and GAPDH B. A selection of MC3T3-E1 RNA samples were used for RT-PCR using each pair of primers followed by gel electrophoretic separation and visualization under UV light. Each primer pair was tested under two experimental conditions and in duplicate to minimize the chance of error as well as the effect of particular experimental conditions on the efficacy of the PCR reaction. PCR products from periostin and

GAPDH primer sets after gel electrophoresis are depicted in Figure 1 and Figure 2. Figure 1 represents the PCR products produced from Periostin A and GAPDH A primers while Figure 2 shows the PCR products from Periostin B and GAPDH B primers. The contents of each lane for the gels shown in Figures 1 and 2 are described in detail in Tables IV and V respectively. For both gels, lane 1 was loaded with a DNA standard ladder, which was used to identify periostin and GAPDH bands based on known sizes, while lane 2 was left empty as a negative control. In Figure 1, increased band intensity was detected in lanes 5, 6, 9 and 10, indicative of a greater PCR yield associated with Periostin A primers. On the other hand, lanes 3, 4, 7 and 8 did not show any definitive bands, which indicated the lack of PCR product generated from GAPDH A primers. Figure 2 shows the results of the second primer sets, Periostin B and GAPDH B. In Figure 2, intense bands were evident in lanes 3, 4, 5 and 6 corresponding to GAPDH B primers while lanes 7, 8, 9 and 10, representing Periostin B primers, did not have any significant bands. From these two figures, it can be concluded that the Periostin A and GAPDH B primers provide the strongest responses, which indicates that these particular primer pairs were the most effective in the RT-PCR reaction for maximum yield. Because the contrast between the different primers was so pronounced and obvious, visual analysis and comparison was sufficient to determine the most effective primers. No other comparison was necessary or conducted. Thus, Periostin A and GAPDH B primers are the most effective and appropriate primers for maximizing the RT-PCR reaction and were the primers chosen for use across all experimental samples.



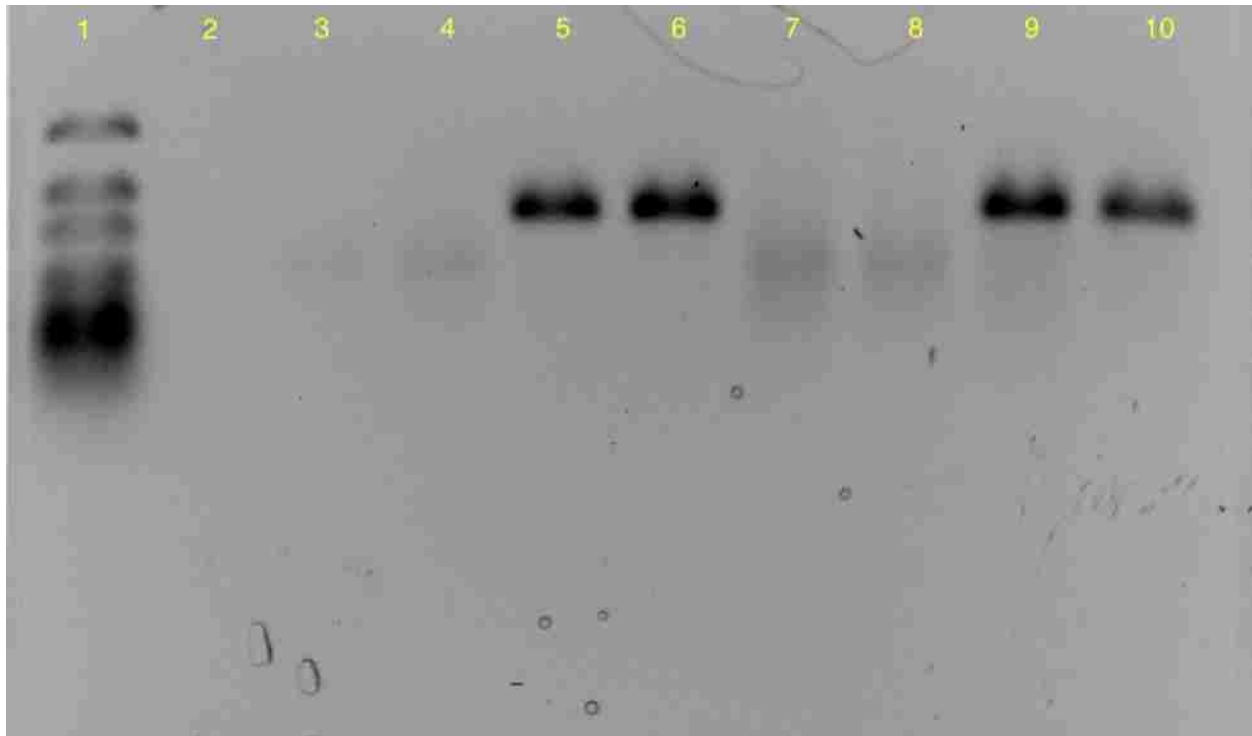


Figure 1. Gel electrophoresis of RT-PCR products using Periostin A and GAPDH A primer sets. Lane 1 contained the DNA standard ladder. Lane 2 was left empty as a negative control. Lanes 3, 4, 7 and 8 contained RT-PCR products generated from GAPDH A primers. Lanes 5, 6, 9 and 10 contained RT-PCR products generated from Periostin A primers. From this figure, it can be concluded that the yields from Periostin A primers (lanes 5, 6, 9, 10) were significant and the yields from GAPDH A (lanes 3, 4, 7, 8) were minimal as indicated by the intensity of the Periostin A bands versus GAPDH A bands.

Lane	Contents/Primers used	Experimental Condition
1	DNA Standard Ladder	N/A
2	Empty	Empty
3	GAPDH A	No BMP
4	GAPDH A	BMP2
5	Periostin A	No BMP
6	Periostin A	BMP2
7	GAPDH A	No BMP
8	GAPDH A	BMP2
9	Periostin A	No BMP
10	Periostin A	BMP2

Table IV. Contents of each lane for the gel electrophoresis of Periostin A and GAPDH A primer sets. Lane 1 contained the DNA standard ladder used to assist in the identification of periostin and GAPDH bands. Lane 2 was left empty as a negative control. All other wells contained Periostin A and GAPDH A primer products under different experimental conditions. Two experimental conditions, selected at random and in duplicate, were used to test each primer to eliminate any experimental error and confounding variables associated with the experimental condition itself (with or without BMP treatment).

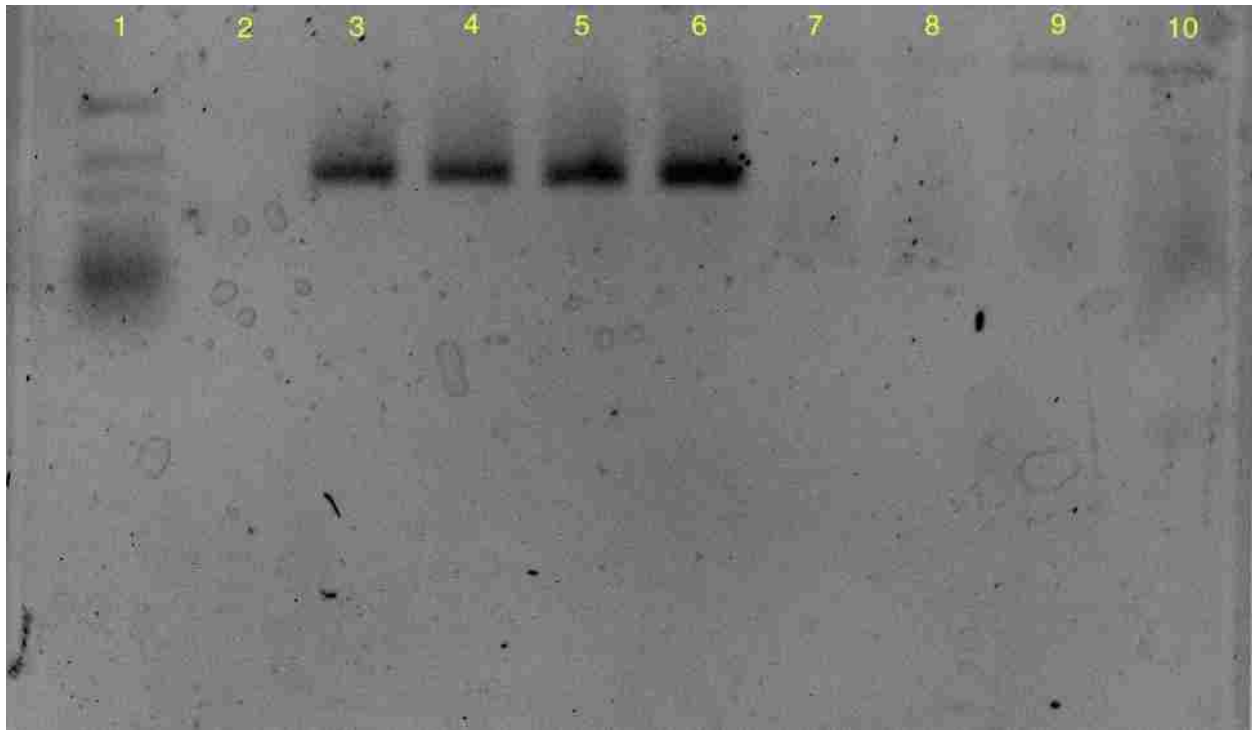


Figure 2. Gel electrophoresis of RT-PCR products using Periostin B and GAPDH B primer sets. Lane 1 contained the DNA standard ladder. Lane 2 was left empty as a negative control. Lanes 3-6 contained RT-PCR products generated from GAPDH B primers. Lanes 7-10 contained RT-PCR products generated from Periostin B primers. From the figure, it can be concluded that the yields from GAPDH B primers were significant and the yields from Periostin B were minimal as indicated by the intensity of the GAPDH B bands versus Periostin B bands.

Lane	Contents/Primers used	Experimental Condition
1	DNA Standard Ladder	N/A
2	Empty	Empty
3	GAPDH B	No BMP
4	GAPDH B	BMP2
5	GAPDH B	No BMP
6	GAPDH B	BMP2
7	Periostin B	No BMP
8	Periostin B	BMP2
9	Periostin B	No BMP
10	Periostin B	BMP2

Table V. Contents of each lane for the gel electrophoresis of Periostin B and GAPDH B primer sets. Lane 1 contained the DNA standard ladder used to assist in the identification of periostin and GAPDH bands. Lane 2 was left empty as a negative control. All other wells contained Periostin B and GAPDH B primer products under different experimental conditions. Two experimental conditions, selected at random and in duplicate, were used to test each primer to eliminate experimental error and any confounding variables associated with the experimental condition itself (with or without BMP treatment).

### 3.2: BMP-Induced Periostin Expression

Periostin expression is known to be up-regulated by TGF- $\beta$ 1 and BMP2 as demonstrated in previous studies (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). These factors are a part of the larger TGF- $\beta$  superfamily of proteins and because of the shared homology, it can be expected that other BMPs would affect periostin expression in some manner. To investigate the effect of BMPs on periostin expression, BMPs with a known role in osteogenesis were selected and applied as treatment conditions to MC3T3-E1 pre-osteoblast cells: BMP2, BMP3, BMP4 and BMP7. After RNA purification, reverse transcription and PCR amplification using specific primers, the amplified cDNA segments were separated on a 2% agarose gel to identify the bands corresponding to the periostin and GAPDH amplified products. Figure 3 is an example of a gel separating periostin amplified products and a gel of GAPDH products derived from the same experimental RNA samples; labeled (A) and (B) respectively. For Figure 3, the contents of each lane are indicated in Table VI. Visual analysis and photography were used to confirm and quantify periostin cDNA bands based on pixel density, which were later normalized against corresponding GAPDH cDNA bands. After normalization, adjusted density values were obtained, which were used to compare individual experiments. Averages in band pixel density were generated as a reflection of the amount of periostin and GAPDH expressed and a student's t-test was used to evaluate significant changes in periostin expression in comparison to the control group (p-value  $\leq$  0.05).

Periostin expression was significantly increased with BMP2 and BMP3 treatments with both low (10 ng/mL) and high (25 ng/mL) concentrations. BMP2 treatment increased periostin expression by 29% and 26% under low and high concentrations, respectively. BMP3 treatment

showed an increase of 24% and 17%, respectively. All values were statistically significant ( $p$ -value  $\leq 0.05$ ). BMP2 treatment showed a larger increase in periostin expression relative to BMP3, reaffirming the powerful osteoinductive nature of BMP2 and confirming the findings from previous studies (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). Collectively, the range of percent change in periostin expression was between 17%-29% for BMP2 and BMP3 treatments. Other BMPs tested, including BMP4 and BMP7, also increased periostin expression under low and high concentrations. For BMP4 treatment, a 9% and 11% increase in periostin expression was observed under low and high concentrations, respectively. BMP7 treatment demonstrated a 5% and 11% increase in periostin expression, respectively. However, these increases in periostin expression by BMP4 and BMP7 were not statistically significant in either low or high concentrations ( $p$ -value  $\leq 0.05$ ). Figure 4 illustrates the changes in periostin expression under each treatment condition and is based on the adjusted density values presented in Table VII. Table VII details the adjusted density values representing changes in periostin expression and consequent t-test values used to determine significance. It was hypothesized that BMP2, BMP4 and BMP7 would significantly increase periostin expression based on previous studies describing the osteoinductive nature of these BMPs (Carreira et al., 2014; Oryan et al., 2014). In addition, BMP3 was expected to reduce periostin expression and behave as an antagonist. This was not observed in our data. Instead, BMP3 increased periostin expression by a significant margin. Overall, periostin expression was enhanced both with low (10ng/mL) and high concentrations (25 ng/mL) of BMP2 and BMP3 only. Although BMP4 and BMP7 also increased periostin expression, this finding was not significant, but at the same time, it does not discount the potential role of these particular BMPs on periostin expression. Taken

together, these findings support the intrinsic ability of the TGF- $\beta$  superfamily of proteins to affect periostin expression to varying degrees.

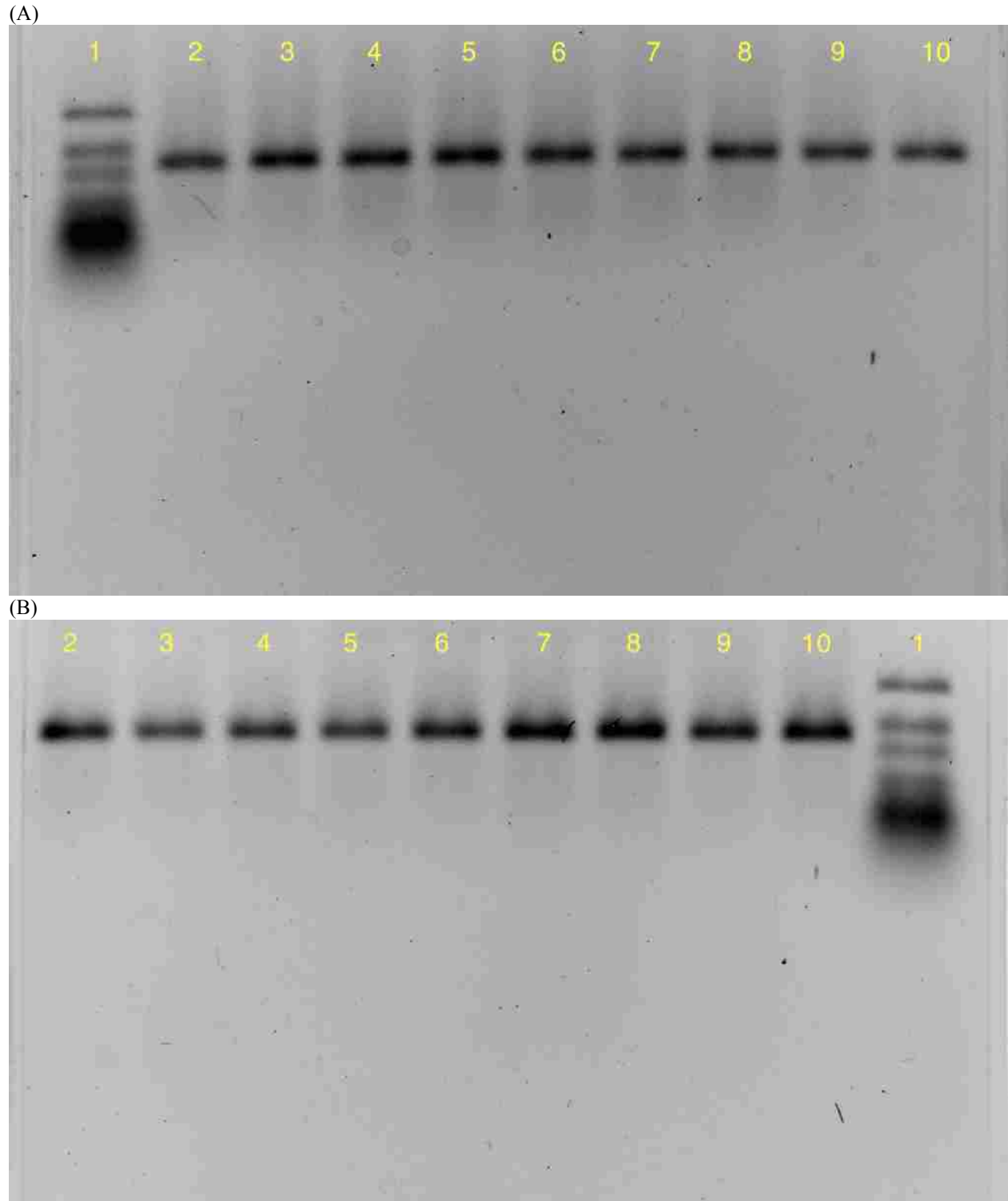


Figure 3. PCR products of periostin and GAPDH primers after gel electrophoresis. Label (A) indicates the gel containing periostin PCR products while label (B) shows the GAPDH PCR products. Lane 1 contained the DNA standard ladder. Lanes 2-10 contained PCR products from different experimental conditions, which are outlined in Table VI. Periostin and corresponding GAPDH bands were identified and quantified for pixel density. To compare across experimental conditions, periostin bands were normalized against the corresponding GAPDH bands, creating adjusted density values. In total, three independent sets of experiments were performed, each with their own periostin and GAPDH paired gels.

Lane	Experimental condition	BMP Concentration (ng/mL)
1	DNA Standard Ladder	N/A
2	Control (no BMP)	0
3	BMP2 (low)	10
4	BMP2 (high)	25
5	BMP3 (low)	10
6	BMP3 (high)	25
7	BMP4 (low)	10
8	BMP4 (high)	25
9	BMP7 (low)	10
10	BMP7 (high)	25

Table VI. Contents of each lane of the gel electrophoresis of periostin and GAPDH PCR products. Lanes are numbered to identify periostin and GAPDH PCR product in the gels shown in Figure 3.

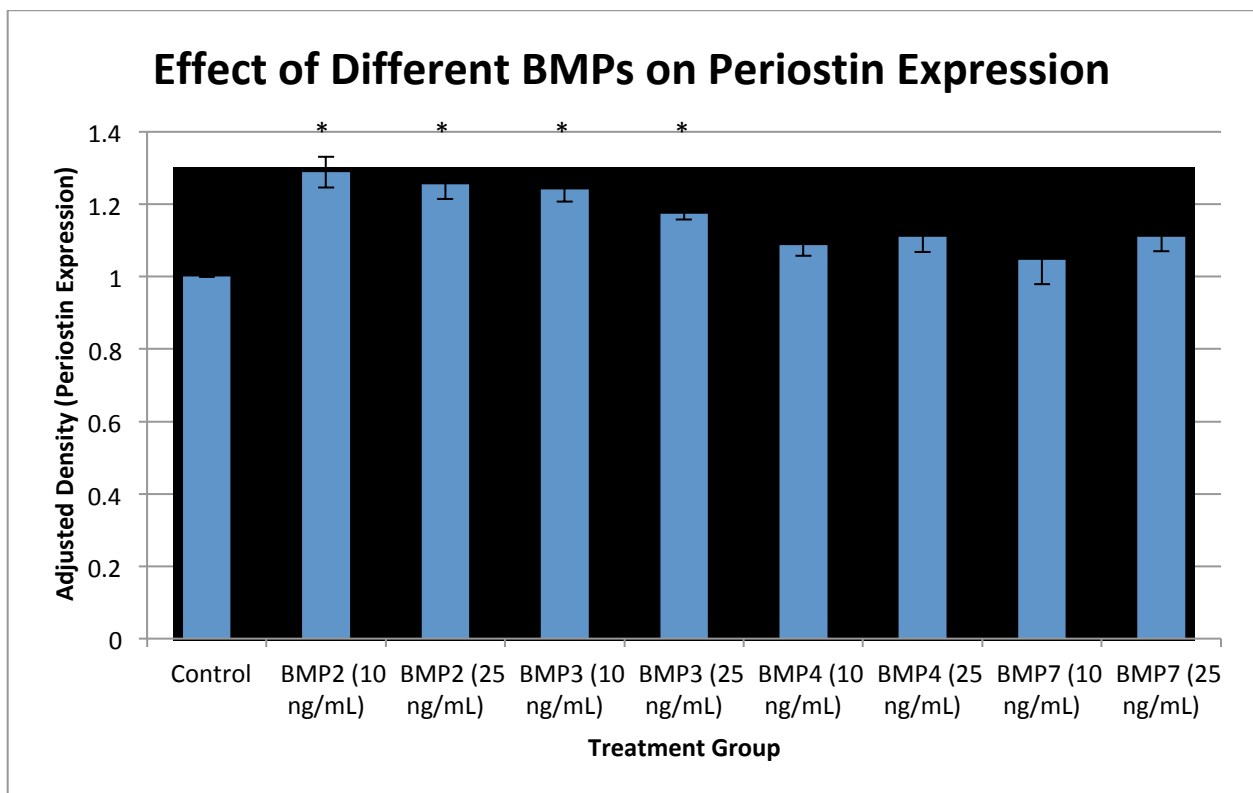


Figure 4. Effect of different BMPs on periostin expression. Periostin expression was normalized against the corresponding GAPDH expression to achieve adjusted values, which could be compared between experiments. Although all BMP treatment groups appeared to increase periostin expression relative to the control group (no BMP), significant increases in periostin expression were only observed with BMP2 and BMP3 at 10 ng/mL and 25 ng/mL. Statistical significance was established at  $p$ -value  $\leq 0.05$ . Significant increases in periostin expression are indicated with \*.

Treatment Group	Adjusted Density	Percent Change (%)	T-Test (BMP to Control)	T-Test (low to high BMP)	Standard Deviation	Standard Error of Mean
Control	1	N/A	N/A	N/A	0	0
BMP2 (10 ng/mL)	1.29	28.8	0.0342*	0.377	0.147	0.0849
BMP2 (25 ng/mL)	1.26	25.5	0.0373*	0.377	0.138	0.0799
BMP3 (10 ng/mL)	1.24	24.0	0.0325*	0.171	0.116	0.0668
BMP3 (25 ng/mL)	1.17	17.3	0.0135*	0.171	0.0555	0.0320
BMP4 (10 ng/mL)	1.09	8.70	0.113	0.201	0.101	0.0581
BMP4 (25 ng/mL)	1.11	11.1	0.134	0.201	0.148	0.0854
BMP7 (10 ng/mL)	1.05	4.60	0.374	0.218	0.231	0.134
BMP7 (25 ng/mL)	1.11	11.1	0.143	0.218	0.140	0.0811

Table VII. Adjusted density values illustrating the changes in periostin expression under different experimental conditions. Increase in periostin expression was significant under the following conditions: BMP2 (10 ng/mL), BMP2 (25 ng/mL), BMP3 (10 ng/mL) and BMP3 (25 ng/mL). Fold change in periostin expression were noted as 29%, 26%, 24% and 17% respectively. Concentration-dependent relationships between BMPs and periostin expression were not observed. Student's t-test was used to determine significance at p-value  $\leq 0.05$ . Standard deviation and standard error of the mean are noted above. Significant increases in periostin expression are indicated with \*.

### 3.3: Concentration-Dependent Relationships between BMP and Periostin Expression

The above findings suggest that members of the TGF- $\beta$  superfamily of proteins specifically, BMPs, do have the ability to influence the expression of periostin. However, one relationship that has not been considered is the concentration-dependent effects of BMPs. In varying the concentrations of the different BMPs of 10 ng/mL and 25 ng/mL, the effect of BMP concentration on periostin expression was evaluated. Using the same experimental protocol, concentration-dependent relationships were evaluated by comparing the adjusted densities, which again reflect periostin expression, between low and high concentrations within the same



BMP group. For each experiment, periostin and GAPDH bands were identified and quantified based on pixel density followed by normalization to generate adjusted density values. Adjusted densities for each treatment group are listed in Table VII. To determine significance, adjusted densities of low versus high concentration of BMPs were compared and contrasted against the control group using a student's t-test with significance set as  $p\text{-value} \leq 0.05$ . For all samples, significant changes in periostin expression as a function of concentration were not observed. In other words, although periostin expression varied depending on concentration, these differences were not significant enough to suggest that concentration-dependent relationships between BMP and periostin were present ( $p\text{-value} \leq 0.05$ ). The relationship between BMP concentration and periostin expression is illustrated in Figure 5 (BMP2), Figure 6 (BMP3), Figure 7 (BMP4) and Figure 8 (BMP7). In each of the figures, different concentrations of BMPs are represented and subsequently compared to determine the significance of concentration on periostin expression. Overall, concentration-dependent relationships in periostin expression were not observed for any of the BMPs.

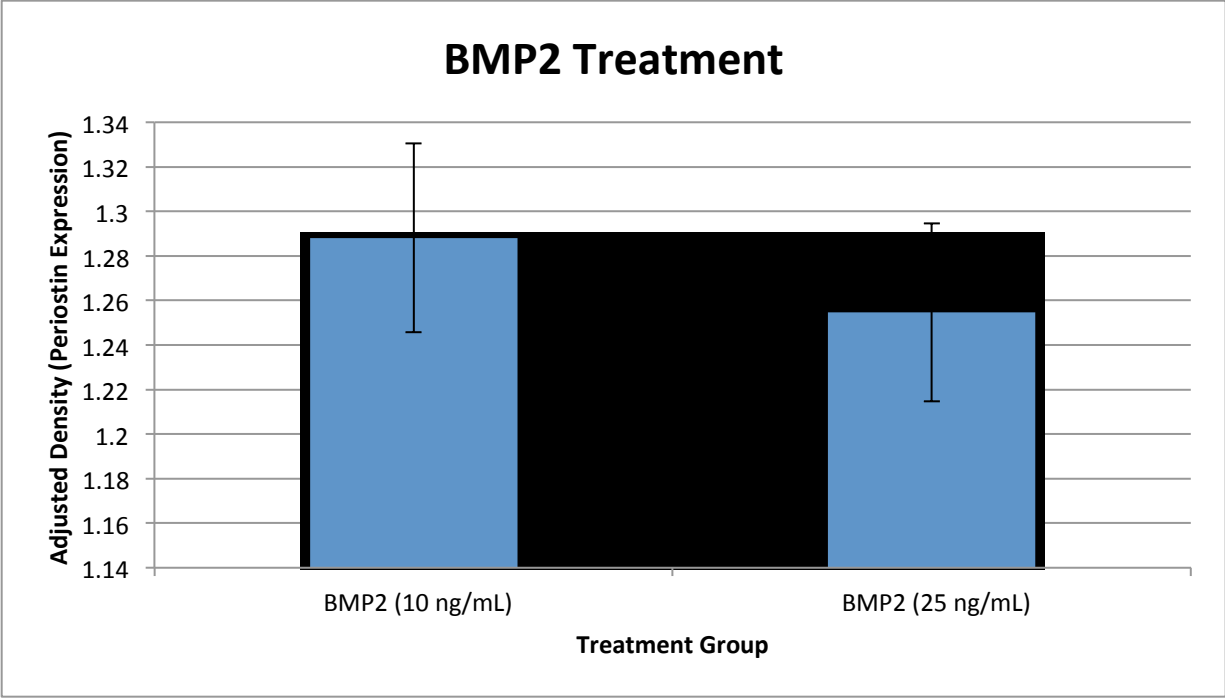


Figure 5. Effect of BMP2 concentration on periostin expression. Although a change in adjusted density, indicative of periostin expression, was observed, this change was not significant ( $p\text{-value} \leq 0.05$ ).

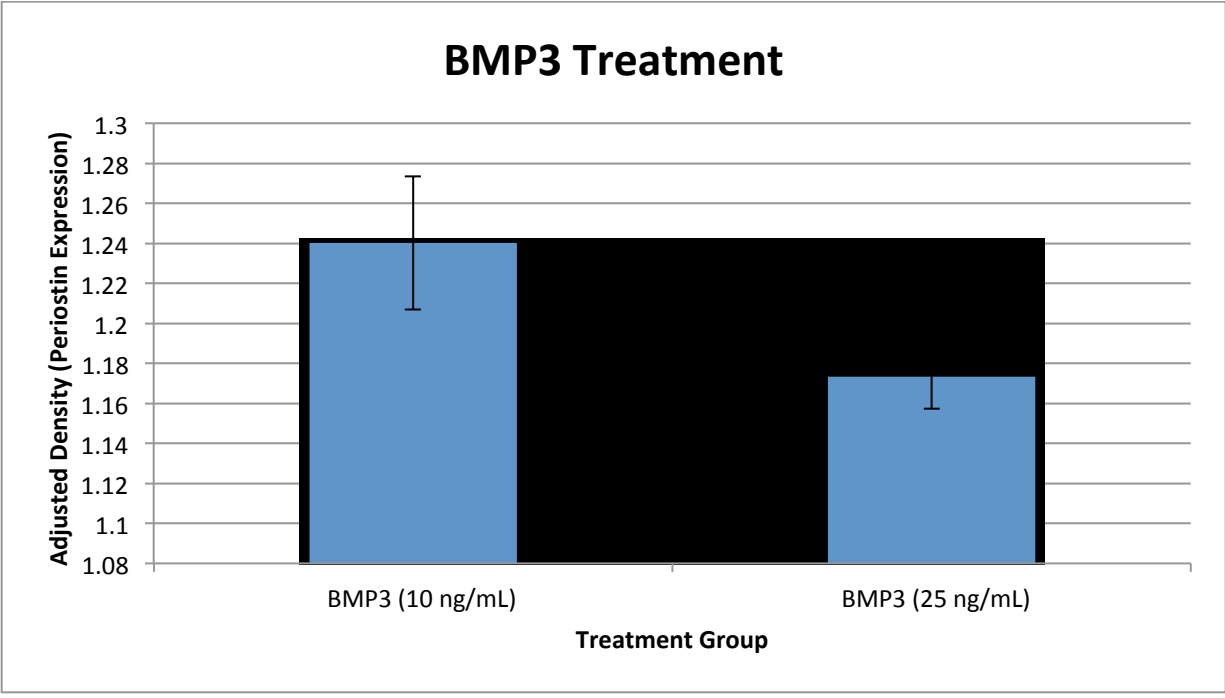


Figure 6. Effect of BMP3 concentration on periostin expression. Although a change in adjusted density, indicative of periostin expression, was observed, this change was not significant ( $p\text{-value} \leq 0.05$ ).

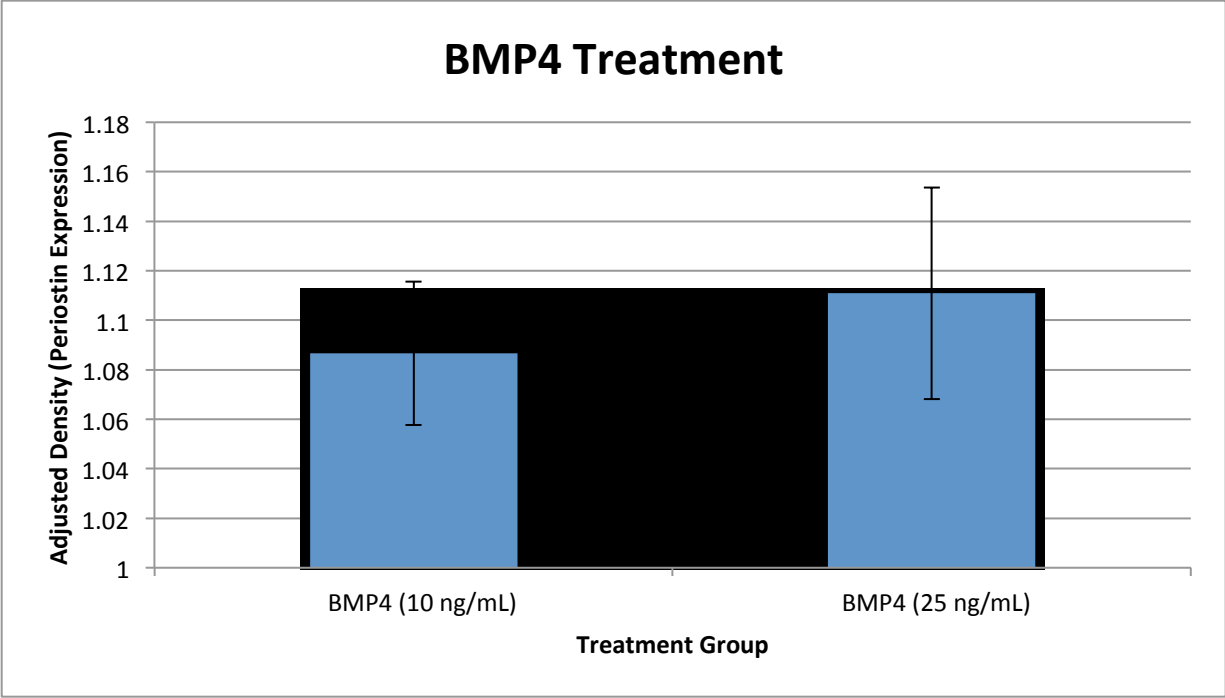


Figure 7. Effect of BMP4 concentration on periostin expression. Although a change in adjusted density, indicative of periostin expression, was observed, this change was not significant ( $p\text{-value} \leq 0.05$ ).

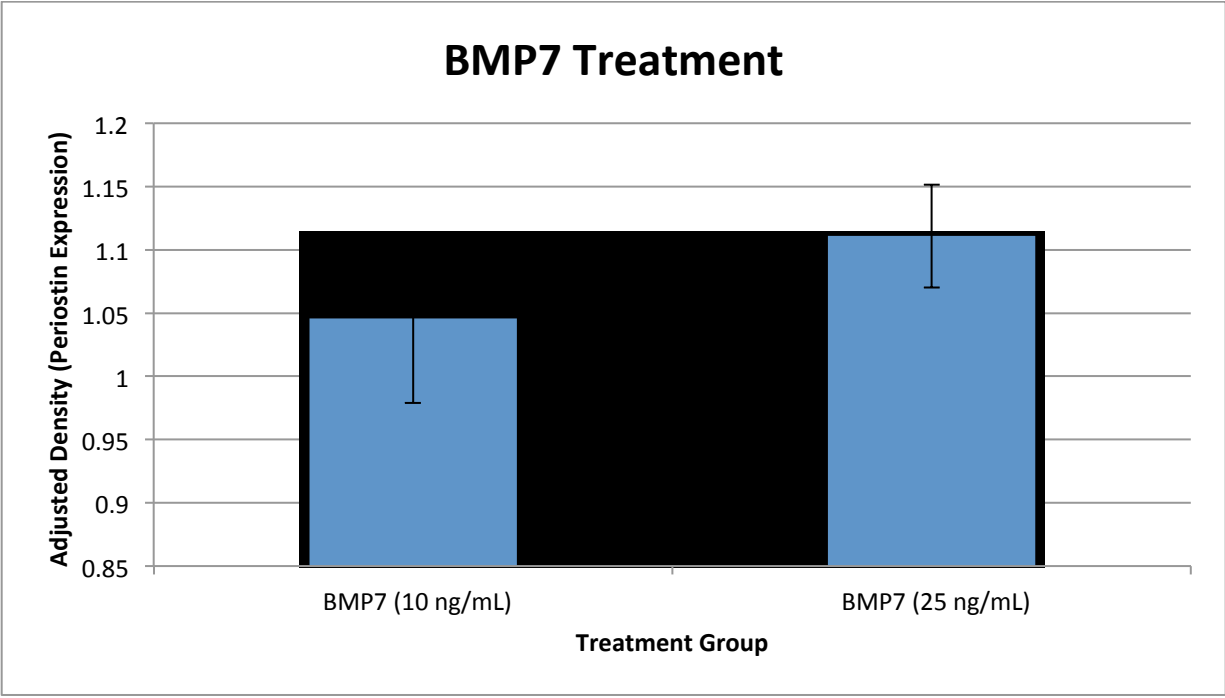


Figure 8. Effect of BMP7 concentration on periostin expression. Although a change in adjusted density, indicative of periostin expression, was observed, this change was not significant ( $p\text{-value} \leq 0.05$ ).

## Chapter 4: Discussion

At the beginning of this thesis, an extensive background on periostin and BMPs was provided as a guide to formulate appropriate research questions and hypotheses as well as direct the purpose of this study. Upon its discovery, periostin was initially identified as a small, secreted 90 kDa glycoprotein with a specific localization towards the periosteum of long bones and the PDL (Horiuchi et al., 1999). We now understand that periostin's expression is ubiquitous throughout the body with an increased expression within collagen fibrous connective tissues of the body, especially within those tissues under mechanical stresses (Nicolas Bonnet et al., 2016; Horiuchi et al., 1999; Shimazaki et al., 2008). More detailed studies uncovered a complex molecular structure to periostin, which served as evidence to help explain the various functions and roles of the protein (Horiuchi et al., 1999; Kudo, 2011; Sugiura et al., 1995). In addition to acting as pillars of support during mechanical stress, periostin has been shown to be involved in many important processes including growth and development, cell adhesion, ECM organization and maintenance, cytodifferentiation, wound healing, inflammation and various pathologies within the body (Kudo, 2017; Kudo & Kii, 2017; Ruan et al., 2009; Yamada et al., 2014). As a key component of so many processes, periostin regulation is paramount and has been demonstrated by many transcription factors, processes and notably, cytokines (B. Merle & Garnero, 2012). One of the most well studied and discussed cytokines are BMPs, members of the TGF- $\beta$  superfamily of proteins. BMPs were first noted for their intrinsic osteoinductive potential on bone formation (Marshall R Urist, 1965). Since then, numerous isoforms of BMPs have been identified and all have crucial roles in physiology and growth and development (Carreira et al., 2014). The tight regulation of BMPs is also important and occurs along both SMAD-dependent and SMAD-independent pathways, which helps control the degree of BMP expression and

ensure that the proper response is elicited (Oryan et al., 2014). From the literature review as a whole, it is clear that periostin and BMPs possess a global influence over the growth and development of an individual. The commonality that brings these two proteins together is their innate ability to govern osteodifferentiation and subsequent bone formation (Horiuchi et al., 1999; Oryan et al., 2014). Previous studies have provided confirmatory evidence of the connection between periostin and BMPs. Specifically, periostin expression is increased in the presence of TGF- $\beta$ 1 and BMP2, which is significant as it suggests the possibility of controlling the expression of one with the other (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). Given the numerous isoforms of BMPs discovered, with each having a differential pattern of expression, the logical question that follows and one which, to our knowledge, has never been asked is: How does periostin expression change with respect to other BMPs? To answer this question, our study examined periostin expression in relation to other BMPs known to play a role in osteogenesis: BMP3, BMP4 and BMP7. BMP2 was also re-examined to confirm the findings from previous studies. To further our understanding of the relationship between periostin expression and BMPs, the effect of concentration was assessed as well.

The present study is the first and only study to examine periostin expression as it relates to the entire gamut of BMPs known to play a role in osteogenesis: BMP2, BMP3, BMP4 and BMP7. To our knowledge, it is also the only known study to consider concentration-dependence as a way to further analyze the relationship between periostin expression and BMPs. Based on the current understanding of osteogenic BMPs, we initially hypothesized that periostin expression would be enhanced by BMP2 (shown previously), BMP4 and BMP7. We also hypothesized that periostin expression would be suppressed by BMP3, given its modulatory type of activity with antagonistic elements. We predicted that any relationship observed between

periostin expression and BMP treatment would be further amplified with increasing concentration, confirming a concentration-dependent type of relationship. Furthermore, we did not anticipate that any of these relationships would be completely reversed by changing the concentrations of BMPs. For instance, if BMP2 was shown to increase periostin expression, we did not expect BMP2 to suddenly decrease periostin expression with changing concentrations. To test these hypotheses, murine cells were treated with different BMPs under varying concentrations followed by RT-PCR and gel electrophoresis to identify and quantify changes in periostin expression.

The underlying objective of this study was to demonstrate an *in vitro* change in periostin expression upon exposure to varying BMPs at different concentrations. MC3T3-E1 pre-osteoblast cells were selected as they are the gold standard murine cell for studying bone biology, as indicated by their use in countless studies on osteodifferentiation and mineralization (Cobo et al., 2016; Lee et al., 2017; Lindner et al., 2005; Blandine Merle et al., 2014). It is an ideal model for studying transcriptional control of osteoblasts for many reasons including cost, convenience, ease of cultivation and its ability to be easily inducible in terms of gene alteration (Wang et al., 1999). The selection of MC3T3-E1 cells is further supported by evidence demonstrating periostin as a marker for the pre-osteoblastic stage of bone cell maturation (Horiuchi et al., 1999). For our purposes, the ability of the MC3T3-E1 cells to undergo effective and rapid changes in periostin gene expression with BMP treatment is critical to ensure that any changes in periostin expression are fully realized as well as to maximize the overall efficiency of the experiments. After BMP treatment, RT-PCR was performed followed by gel electrophoretic separation to quantify, compare and contrast the degree of periostin expression between the treatment groups. A comparison of specific primers to periostin and GAPDH, a housekeeping

gene, were necessary to optimize the RT-PCR experimental conditions. Each set of primers served a unique purpose in this study to ensure the validity of our results. Periostin primers were utilized to amplify a portion of the periostin cDNA. This provided an assessment of up-regulation, down-regulation or perhaps even no change, depending on periostin's transcriptional response to the different BMP treatments. In a similar manner, GAPDH primers were employed to amplify a part of the GAPDH cDNA, which acts as a baseline measure for the total number of cells in each experiment. As a housekeeping gene, GAPDH is constitutively active in all live cells and thus, reflects the total cell count (Lee et al., 2017; Blandine Merle et al., 2014). The assumption is that cell numbers between each of the experiments varies to a certain degree and consequently, in order to compare the actual periostin expression between the experiments, a normalization to cell number must be considered. If this normalization is not performed, then any changes in periostin expression could be attributed to differences in cell number, which would weaken any claims of association between periostin expression and BMPs.

In the first part of our study, different periostin and GAPDH primers were evaluated to determine whether different primer sets demonstrated significant differences in efficiency during RT-PCR. In theory, the most complementary primer sequences to the target gene should possess the greatest binding affinity and therefore, bear the most efficient transcription for the corresponding gene. Despite their conserved gene sequences, there are still a plethora of potential periostin and GAPDH primers for RT-PCR, each with different primer sequences and presumptively, transcriptional efficiencies. To determine the most ideal primers, two different primer sets for periostin and GAPDH were drawn from the literature and tested. Selecting from recent literature increases the probability that the primers selected would be robust and capable of efficient amplification, since they would have already been successfully utilized in other

studies (Lee et al., 2017; Matsuzawa et al., 2015; Blandine Merle et al., 2014; Tilman et al., 2007). A consideration was given to include more than two primer sets for both periostin and GAPDH to be as comprehensive as possible. However, it was decided that two primer sets, especially if taken from the literature and utilized in previous studies, would be adequate, especially considering other aspects such as cost, time and simplification of the overall methodology. Unsurprisingly, some periostin and GAPDH sets were more efficient than others for RT-PCR. Despite being key proteins that are evolutionarily conserved across species, periostin and GAPDH gene sequences cannot be expected to be 100% conserved given individual variations in the genetic code, genetic mutations and splice variations (Hoersch & Andrade-Navarro, 2010a; Horiuchi et al., 1999; Seidler, 2013). For all of these reasons, it was expected that some primers would perform better than others and this was observed. However, since these primer sets were taken from the literature, we did not expect such a wide discrepancy in RT-PCR efficiency. For both periostin and GAPDH, the poorly performing primer did not show any definitive bands, indicating that the specific amplification did not occur at all. This finding could be attributed to variations in the DNA sequence of the MC3T3-E1 subclones giving rise to an ineffective primer-gene association. Nevertheless, once the most efficient primers were identified, these primers were employed to examine the effect of BMPs on periostin expression.

In the second and main part of our study, the effect of varying concentrations of different BMPs on periostin expression was examined. The results of our study showed mixed findings, some congruent and others incongruent with our original hypotheses. Predictably, BMP2 was found to significantly increase periostin expression by 29% and 26% under low (10 ng/mL) and high (25 ng/mL) concentrations respectively relative to the control (no BMP), confirming



previous findings within the literature (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). Interestingly, BMP3 was also found to increase periostin expression by 24% and 17% under low and high concentrations respectively, which was not anticipated. No statistically significant increase in periostin expression was observed with BMP4 or BMP7. BMP2 has been extensively studied and has been reliably shown to enhance periostin expression on many occasions (Horiuchi et al., 1999; Ji et al., 2000; Blandine Merle et al., 2014). Findings from our study complement the growing body of knowledge surrounding BMP2 and its powerful osteoinductive properties, paving the way for a greater understanding of the relationship between these osteogenic proteins (Ali & Brazil, 2014; Oryan et al., 2014). Examining periostin and BMP2 closer reveals many overlapping functions in ECM organization, osteodifferentiation and osteogenesis (Ali & Brazil, 2014; Horiuchi et al., 1999; Kudo, 2011; Kudo & Kii, 2017; Oryan et al., 2014). Horiuchi *et al.* (1999) showed that periostin expression is enhanced until the point when mature osteoblasts appear and bone mineralization begins, suggesting that periostin may be involved in initiating osteodifferentiation (Horiuchi et al., 1999). As an integral component to osteogenesis, BMP2 plays a significant role in ensuring that osteodifferentiation and bone formation continue towards completion (Oryan et al., 2014). Taken together, it is likely that the functions of periostin and BMP2 are intertwined to coordinate the initiation and propagation of osteoblast differentiation and bone formation processes, underscoring the dynamic interplay between these proteins (Horiuchi et al., 1999; Oryan et al., 2014). Contrary to our hypothesis, we observed a significant increase in periostin expression with BMP3. In knowing the association between periostin and osteodifferentiation, we hypothesized that BMP3 would suppress periostin expression given its powerful inhibitory nature on osteogenesis (Daluisi et al., 2001; Gamer, Nove, Levin, & Rosen, 2005). BMP3 has even been demonstrated to be capable of reversing the

differentiation of cells already destined to become osteoblasts, further emphasizing the overwhelming osteosuppressive potential of BMP3 (Kokabu et al., 2012). Despite all of the evidence of BMP3 as an osteoinhibitor, there has been some evidence that suggests otherwise. BMP3 has, in fact, been shown to elicit modulatory activity in fracture and wound healing and be a keystone to bone homeostasis (Chang et al., 2012). This evidence goes against the conventional thinking of BMP3 as an inhibitor to bone differentiation and suggests that BMP3 may not be purely inhibitory. In considering the findings by Chang *et al.* (2012) and our own, we postulate that BMP3 has the potential to be inhibitory or modulatory depending upon external factors and the surrounding conditions, which may alter its response (Chang et al., 2012). Unlike BMP3, there has been conclusive evidence of BMP4 and BMP7 as osteogenic factors responsible for inducing osteoblast differentiation and subsequent bone formation, which makes our findings all the more surprising (Franceschi et al., 2000; Nishimura et al., 2012; W. Zhu et al., 2004). Periostin, like many key proteins, is regulated by several external controllers, any of which could have played a role in concealing the true effect of BMP4 and BMP7 on periostin expression. In addition, the lack of periostin expression could have also been the result of less than optimum concentrations of BMPs. At the current concentrations, our results suggest that BMP2 and BMP3 affect periostin expression, but it is possible that this osteoblast model was not sensitive enough to detect and respond to BMP4 and BMP7 at the prescribed concentrations. In other words, concentrations of BMP4 and BMP7 may have been too low to induce a periostin response. Furthermore, in all cases, periostin expression was evaluated after 24 hours of incubation. Although this time frame may be adequate in a majority cases, extending the incubation period would increase the time that BMPs are exposed to the cells, which may have an effect on periostin expression.

In the final part of our study, the effect of concentration-dependence was evaluated to determine whether varying the concentrations of BMPs could change periostin expression. From our findings, we found that there was no difference in periostin expression at the concentrations tested. For each of the BMPs, two concentrations were selected and used to treat the cells: 10 ng/mL (low) and 25 ng/mL (high). The concentrations selected have been shown to be effective in other studies on cytokines, which makes their selections appropriate (Horiuchi et al., 1999; Blandine Merle et al., 2014). There are many possibilities to explain the observations in our study. As previously mentioned, cells may not have been sensitive enough to BMP4 and BMP7 to elicit a change in periostin expression. Increasing the concentration of BMPs would be an ideal technique to fully realize the effect of concentration-dependence on periostin expression. Furthermore, a wider range of concentrations, perhaps from 10 ng/mL to 100 ng/mL, should also be considered to fully appreciate the effect of BMP concentration on periostin expression and establish any dose-dependent relationships. By incorporating higher concentrations and expanding the range of concentrations, the complete relationship between periostin expression and concentration-dependence can be established, which may be important on a therapeutic level in the future. Although the effect of concentration was not observed in our study, concentration-dependent relationships may very well exist at concentrations outside of our range and will need to be further examined in future studies.

The FDA has approved BMP2 and BMP7 to be used therapeutically in their recombinant forms as rhBMP2 and rhBMP7 respectively (Carreira et al., 2014). To date, rhBMP2 and rhBMP7 have widespread acceptance and have been demonstrated to be beneficial in orthopedics, dentistry and wound healing (Carreira et al., 2014). Given the interrelated functions between periostin and BMPs, it is probable that periostin is involved in these observations,

working in tandem with BMPs to facilitate bone formation, although this notion has yet to be proven. Specifically, periostin may act as an initiator of osteodifferentiation and synergize with BMPs to accelerate osteodifferentiation and subsequent bone formation (Horiuchi et al., 1999). In relation to dentistry and in particular, orthodontics, periostin expression has been demonstrated to be increased on the compressive side of a tooth during orthodontic tooth movement (OTM) and during hypoxia (P. Li et al., 2004; Wilde et al., 2003). Moreover, periostin is a ligand for  $\alpha\beta3$  and  $\alpha\beta5$  integrins and promotes integrin-dependent cell adhesion, which may be critical for the osteodifferentiation and migration of osteoblasts during OTM (Cobo et al., 2016; Conway et al., 2014). With shared roles in bone remodeling, the joint application of periostin and BMPs may grant increased therapeutic benefits, not only in OTM, but also in orthopedics, dentistry and wound healing. Within orthodontics, clinical benefits from periostin and BMP treatment may include enhanced treatment results, accelerated treatment times and increased overall patient satisfaction. In orthopedics and wound healing, enhanced bone remodeling may translate to improved wound and fracture healing as well as shorter durations of treatment. Thus, the functions and roles of periostin and BMPs appear to be linked, and together, their combination and synergy may equate to therapeutic improvements across many disciplines. One caveat to consider would be the high anticipated costs, since recombinant periostin would need to be produced and thoroughly tested to ensure safety prior to clinical use. Further research with an *in vivo* model is necessary to examine the relationship between periostin and BMPs and determine whether the therapeutic benefits, if present, are substantial enough to warrant pharmaceutical consideration.

Our study evaluated periostin expression in response to different BMPs under various concentrations. However, there are certain limitations to our study. For one, our study evaluated

each of the BMPs independently and in an *in vitro* setting, which may not be completely valid *in vivo*. We also utilized only two concentrations of BMPs, a low and a high concentration, and followed the experiments for 24 hours, both of which could have limited the total amount of periostin expression possible. To build upon our research, higher concentrations of BMPs with a wider range can be employed to define any concentration-dependent relationships. The effect of time can also be included to realize any time-dependent relationships as well. Given that gene regulation is typically multi-factorial, it would be interesting to examine periostin expression using the BMPs in combination. Treating cells with BMPs in combination would more closely follow gene regulation on a physiological level, as it is rare for any gene to be regulated by only a single factor. In regard to visualization and quantification, periostin gene expression can be measured using other methods, which may potentially be more sensitive and accurate. These methods include Northern blotting, real-time PCR (qPCR) and fluorescent *in situ* hybridization. Western blotting can also be considered and would be an ideal way to measure the amount of periostin RNA that is fully translated into the final, functional protein. As the research on periostin and BMPs continues to grow and expand, it is likely that other factors affecting periostin expression will come into play and these factors need to be considered as well. To date, most of the experiments examining periostin expression and BMPs have been performed *in vitro*, which does not always translate to an *in vivo* setting. Future research should also focus on establishing an *in vivo* experimental model for external validity and applicability or at the very least, employ as many physiologic elements as possible to mimic an *in vivo* setting. In doing so, the relationship between periostin expression and BMPs can be more fully understood for what it really is in nature.

## Chapter 5: Conclusion

1. Periostin expression is increased by BMP2 in MC3T3-E1 pre-osteoblast cells by 29% and 26% under low (10 ng/mL) and high (25 ng/mL) concentrations respectively.
2. Periostin expression is increased by BMP3 in MC3T3-E1 pre-osteoblast cells by 24% and 17% under low (10 ng/mL) and high (25 ng/mL) concentrations respectively.
3. Periostin expression is unaffected by BMP4 and BMP7 in MC3T3-E1 pre-osteoblast cells at any concentration.
4. Concentration-dependent relationships were not observed between periostin expression and any of the tested BMPs within the concentrations examined.

## References

- Ali, I. H. A., & Brazil, D. P. (2014). Bone morphogenetic proteins and their antagonists: Current and emerging clinical uses. *British Journal of Pharmacology*.  
<https://doi.org/10.1111/bph.12724>
- Aoki, H., Fujii, M., Imamura, T., Yagi, K., Takehara, K., Kato, M., & Miyazono, K. (2001). Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. *Journal of Cell Science*, *114*(8), 1483–1489.
- Aro, H. T., Govender, S., Patel, A. D., Hernigou, P., Perera de Gregorio, A., Popescu, G. I., ... Valentin, A. (2011). Recombinant human bone morphogenetic protein-2: a randomized trial in open tibial fractures treated with reamed nail fixation. *The Journal of Bone and Joint Surgery. American Volume*, *93*(9), 801–808. <https://doi.org/10.2106/JBJS.I.01763>
- Arrabal, P. M., Visser, R., Santos-Ruiz, L., Becerra, J., & Cifuentes, M. (2013). Osteogenic molecules for clinical applications: Improving the BMP-collagen system. *Biological Research*. <https://doi.org/10.4067/S0716-97602013000400013>
- Bao, S., Ouyang, G., Bai, X., Huang, Z., Ma, C., Liu, M., ... Wang, X. F. (2004). Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway. *Cancer Cell*, *5*(4), 329–339. [https://doi.org/10.1016/S1535-6108\(04\)00081-9](https://doi.org/10.1016/S1535-6108(04)00081-9)
- Beederman, M., Lamplot, J. D., Nan, G., Wang, J., Liu, X., Yin, L., ... He, T.-C. (2013). BMP signaling in mesenchymal stem cell differentiation and bone formation \*. *J. Biomedical Science and Engineering*, *6*, 32–52. <https://doi.org/10.4236/jbise.2013.68A1004>
- Beertsen, W. (1975). Migration of fibroblasts in the periodontal ligament of the mouse incisor as revealed by autoradiography. *Archives of Oral Biology*, *20*(10).  
[https://doi.org/10.1016/0003-9969\(75\)90134-X](https://doi.org/10.1016/0003-9969(75)90134-X)
- Boerckel, J. D., Kolambkar, Y. M., Dupont, K. M., Uhrig, B. A., Phelps, E. A., Stevens, H. Y., ... Guldberg, R. E. (2011). Effects of protein dose and delivery system on BMP-mediated bone regeneration. *Biomaterials*, *32*(22), 5241–5251.  
<https://doi.org/10.1016/j.biomaterials.2011.03.063>
- Bonnet, N., Conway, S. J., & Ferrari, S. L. (2012). Regulation of beta catenin signaling and parathyroid hormone anabolic effects in bone by the extracellular matrix protein periostin. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(37), 15048–15053. <https://doi.org/10.1073/pnas.1203085109>
- Bonnet, N., Garnero, P., & Ferrari, S. (2016). Periostin action in bone. *Molecular and Cellular Endocrinology*, *432*, 75–82. <https://doi.org/10.1016/j.mce.2015.12.014>

- Bonnet, N., Standley, K. N., Bianchi, E. N., Stadelmann, V., Foti, M., Conway, S. J., & Ferrari, S. L. (2009). The matricellular protein periostin is required for sost inhibition and the anabolic response to mechanical loading and physical activity. *Journal of Biological Chemistry*, *284*(51), 35939–35950. <https://doi.org/10.1074/jbc.M109.060335>
- Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., & Nohe, A. (2011). Bone Morphogenetic Proteins: A critical review. *Cellular Signalling*. <https://doi.org/10.1016/j.cellsig.2010.10.003>
- Carreira, A. C., Lojudice, F. H., Halcsik, E., Navarro, R. D., Sogayar, M. C., & Granjeiro, J. M. (2014). Bone morphogenetic proteins: facts, challenges, and future perspectives. *Journal of Dental Research*, *93*(4), 335–45. <https://doi.org/10.1177/0022034513518561>
- Chang, X., Lu, Y., Shibata, Y., Tsukazaki, T., & Yamaguchi Dr., A. (2012). Role of bone morphogenetic proteins and their antagonists during fracture healing. *Journal of Hard Tissue Biology*, *21*(2). <https://doi.org/10.2485/jhtb.21.203>
- Chen, D., Harris, M. A., Rossini, G., Dunstan, C. R., Dallas, S. L., Feng, J. Q., ... Harris, S. E. (1997). Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. *Calcified Tissue International*, *60*(3), 283–290. <https://doi.org/10.1007/s002239900230>
- Chen, F., Bi, D., Cao, G., Cheng, C., Ma, S., Liu, Y., & Cheng, K. (2017). Bone morphogenetic protein 7-transduced human dermal-derived fibroblast cells differentiate into osteoblasts and form bone in vivo. *Connective Tissue Research*, *0*(0), 1–10.
- Cobo, T., Vilorio, C. G., Solares, L., Fontanil, T., González-Chamorro, E., De Carlos, F., ... Obaya, A. J. (2016). Role of periostin in adhesion and migration of bone remodeling cells. *PLoS ONE*, *11*(1). <https://doi.org/10.1371/journal.pone.0147837>
- Connerney, J., Andreeva, V., Leshem, Y., Muentener, C., Mercado, M. A., & Spicer, D. B. (2006). Twist1 dimer selection regulates cranial suture patterning and fusion. *Developmental Dynamics*, *235*(5), 1345–1357. <https://doi.org/10.1002/dvdy.20717>
- Conway, S. J., Izuhara, K., Kudo, Y., Litvin, J., Markwald, R., Ouyang, G., ... Kudo, A. (2014). The role of periostin in tissue remodeling across health and disease. *Cellular and Molecular Life Sciences : CMLS*. <https://doi.org/10.1007/s00018-013-1494-y>
- Coutu, D. L., Jian, H. W., Monette, A., Rivard, G. É., Blostein, M. D., & Galipeau, J. (2008). Periostin, a member of a novel family of vitamin K-dependent proteins, is expressed by mesenchymal stromal cells. *Journal of Biological Chemistry*, *283*(26), 17991–18001. <https://doi.org/10.1074/jbc.M708029200>



- Daluiski, A., Engstrand, T., Bahamonde, M. E., Gamer, L. W., Agius, E., Stevenson, S. L., ... Lyons, K. M. (2001). Bone morphogenetic protein-3 is a negative regulator of bone density. *Nature Genetics*, 27(1), 84–88. <https://doi.org/10.1038/83810>
- Deschaseaux, F., Sensébé, L., & Heymann, D. (2009). Mechanisms of bone repair and regeneration. *Trends in Molecular Medicine*. <https://doi.org/10.1016/j.molmed.2009.07.002>
- Du, J., & Li, M. (2017). Functions of Periostin in dental tissues and its role in periodontal tissues' regeneration. *Cellular and Molecular Life Sciences*. <https://doi.org/10.1007/s00018-017-2645-3>
- Eijken, M., Swagemakers, S., Koedam, M., Steenbergen, C., Derkx, P., Uitterlinden, A. G., ... van Leeuwen, J. P. T. M. (2007). The activin A-follistatin system: potent regulator of human extracellular matrix mineralization. *The FASEB Journal*, 21(11), 2949–2960. <https://doi.org/10.1096/fj.07-8080com>
- Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M., & Goodman, C. S. (1990). Genetic analysis of a drosophila neural cell adhesion molecule: Interaction of fasciclin I and abelson tyrosine kinase mutations. *Cell*, 60(4), 565–575. [https://doi.org/10.1016/0092-8674\(90\)90660-7](https://doi.org/10.1016/0092-8674(90)90660-7)
- Elliott, C. G., Wang, J., Guo, X., Xu, S. -w., Eastwood, M., Guan, J., ... Hamilton, D. W. (2012). Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair. *Journal of Cell Science*, 125(1), 121–132. <https://doi.org/10.1242/jcs.087841>
- Fan, Z., Chubinskaya, S., Rueger, D. C., Bau, B., Haag, J., & Aigner, T. (2004). Regulation of anabolic and catabolic gene expression in normal and osteoarthritic adult human articular chondrocytes by osteogenic protein-1. *Clin Exp Rheumatol*, 22(1), 103–106. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/15005012>
- Fourman, M. S., Borst, E. W., Bogner, E., Rozbruch, S. R., & Fragomen, A. T. (2014). Recombinant human BMP-2 increases the incidence and rate of healing in complex ankle arthrodesis. *Clinical Orthopaedics and Related Research*, 472(2), 732–739. <https://doi.org/10.1007/s11999-013-3261-7>
- Franceschi, R. T., Wang, D., Krebsbach, P. H., & Rutherford, R. B. (2000). Gene therapy for bone formation: In vitro and in vivo osteogenic activity of an adenovirus expressing BMP7. *Journal of Cellular Biochemistry*, 78(3), 476–486. [https://doi.org/10.1002/1097-4644\(20000901\)78:3<476::AID-JCB12>3.0.CO;2-5](https://doi.org/10.1002/1097-4644(20000901)78:3<476::AID-JCB12>3.0.CO;2-5)
- Fukushima, N., Kikuchi, Y., Nishiyama, T., Kudo, A., & Fukayama, M. (2008). Periostin deposition in the stroma of invasive and intraductal neoplasms of the pancreas. *Modern Pathology*, 21(8), 1044–1053. <https://doi.org/10.1038/modpathol.2008.77>
- Gamer, L. W., Cox, K., Carlo, J. M., & Rosen, V. (2009). Overexpression of BMP3 in the developing skeleton alters endochondral bone formation resulting in spontaneous rib

- fractures. *Developmental Dynamics*, 238(9), 2374–2381.  
<https://doi.org/10.1002/dvdy.22048>
- Gamer, L. W., Nove, J., Levin, M., & Rosen, V. (2005). BMP-3 is a novel inhibitor of both activin and BMP-4 signaling in *Xenopus* embryos. *Developmental Biology*, 285(1), 156–168. <https://doi.org/10.1016/j.ydbio.2005.06.012>
- Garrison, K. R., Shemilt, I., Donell, S., Ryder, J. J., Mugford, M., Harvey, I., ... Alt, V. (2010). BMP for fracture healing in adults Garrison Cochrane Review. *Cochrane Database Syst Rev*, (6). <https://doi.org/10.1002/14651858.CD006950.pub2.www.cochranelibrary.com>
- Govender, S., Csimma, C., Genant, H. K., Valentin-Opran, A., Amit, Y., Arbel, R., ... BMP-2 Evaluation in Surgery for Tibial Trauma (BESTT) Study Group, T. (2002). Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *The Journal of Bone and Joint Surgery. American Volume*, 84–A(12), 2123–34. <https://doi.org/10.1097/00003086-199706000-00008>
- Haertel-Wiesmann, M., Liang, Y., Fantl, W. J., & Williams, L. T. (2000). Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. *Journal of Biological Chemistry*, 275(41), 32046–32051. <https://doi.org/10.1074/jbc.M000074200>
- Hakuno, D., Kimura, N., Yoshioka, M., Mukai, M., Kimura, T., Okada, Y., ... Fukuda, K. (2010). Periostin advances atherosclerotic and rheumatic cardiac valve degeneration by inducing angiogenesis and MMP production in humans and rodents. *Journal of Clinical Investigation*, 120(7), 2292–2306. <https://doi.org/10.1172/JCI40973>
- Hoersch, S., & Andrade-Navarro, M. A. (2010). Periostin shows increased evolutionary plasticity in its alternatively spliced region. *BMC Evolutionary Biology*, 10, 30. <https://doi.org/10.1186/1471-2148-10-30>
- Hoersch, S., & Andrade-Navarro, M. A. (2010). Periostin shows increased evolutionary plasticity in its alternatively spliced region. *BMC Evolutionary Biology*, 10(1). <https://doi.org/10.1186/1471-2148-10-30>
- Hong, P., Boyd, D., Beyea, S. D., & Bezuhy, M. (2013). Enhancement of bone consolidation in mandibular distraction osteogenesis: A contemporary review of experimental studies involving adjuvant therapies. *Journal of Plastic, Reconstructive and Aesthetic Surgery*. <https://doi.org/10.1016/j.bjps.2013.03.030>
- Horiuchi, K., Amizuka, N., Takeshita, S., Takamatsu, H., Katsuura, M., Ozawa, H., ... Kudo, A. (1999). Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, 14(7), 1239–1249. <https://doi.org/10.1359/jbmr.1999.14.7.1239>

- Hussein, K. A., Zakhary, I. E., Hailat, D., Elrefai, R., Sharawy, M., & Elsalanty, M. E. (2013). Delayed versus immediate reconstruction of mandibular segmental defects using recombinant human bone morphogenetic protein 2/absorbable collagen sponge. *Journal of Oral and Maxillofacial Surgery*, *71*(6), 1107–1118. <https://doi.org/10.1016/j.joms.2012.12.018>
- Ishibashi, O., Ikegame, M., Takizawa, F., Yoshizawa, T., Moksed, M. A., Iizawa, F., ... Kawashima, H. (2010). Endoglin is involved in BMP-2-induced osteogenic differentiation of periodontal ligament cells through a pathway independent of Smad-1/5/8 phosphorylation. *Journal of Cellular Physiology*, *222*(2), 465–473. <https://doi.org/10.1002/jcp.21968>
- Izuhara, K., Arima, K., Ohta, S., Suzuki, S., Inamitsu, M., & Yamamoto, K. (2014). Periostin in Allergic Inflammation. *Allergology International*, *63*(2), 143–151. <https://doi.org/10.2332/allergolint.13-RAI-0663>
- Jackson-Boeters, L., Wen, W., & Hamilton, D. W. (2009). Periostin localizes to cells in normal skin, but is associated with the extracellular matrix during wound repair. *Journal of Cell Communication and Signaling*, *3*(2), 125–133. <https://doi.org/10.1007/s12079-009-0057-3>
- Jain, A., Pundir, S., & Sharma, A. (2013). Bone morphogenetic proteins: The anomalous molecules. *Journal of Indian Society of Periodontology*, *17*(5), 583. <https://doi.org/10.4103/0972-124X.119275>
- Ji, X., Chen, D., Xu, C., Harris, S. E., Mundy, G. R., & Yoneda, T. (2000). Patterns of gene expression associated with BMP-2-induced osteoblast and adipocyte differentiation of mesenchymal progenitor cell 3T3-F442A. *J Bone Miner Metab*, *18*(3), 132–139. <https://doi.org/10.1007/s007740050103>
- Johansson, M. W., Annis, D. S., & Mosher, D. F. (2013).  $\alpha 5 \beta 1$  integrin-mediated adhesion and motility of IL-5-stimulated eosinophils on periostin. *American Journal of Respiratory Cell and Molecular Biology*, *48*(4), 503–510. <https://doi.org/10.1165/rcmb.2012-0150OC>
- Kashima, T. G., Nishiyama, T., Shimazu, K., Shimazaki, M., Kii, I., Grigoriadis, A. E., ... Kudo, A. (2009). Periostin, a novel marker of intramembranous ossification, is expressed in fibrous dysplasia and in c-Fos-overexpressing bone lesions. *Human Pathology*, *40*(2), 226–237. <https://doi.org/10.1016/j.humpath.2008.07.008>
- Kii, I., Nishiyama, T., & Kudo, A. (2016). Periostin promotes secretion of fibronectin from the endoplasmic reticulum. *Biochemical and Biophysical Research Communications*, *470*(4), 888–893. <https://doi.org/10.1016/j.bbrc.2016.01.139>
- Kii, I., Nishiyama, T., Li, M., Matsumoto, K. I., Saito, M., Amizuka, N., & Kudo, A. (2010). Incorporation of tenascin-C into the extracellular matrix by periostin underlies an extracellular meshwork architecture. *Journal of Biological Chemistry*, *285*(3), 2028–2039. <https://doi.org/10.1074/jbc.M109.051961>

- Kim, J. E., Kim, S. J., Lee, B. H., Park, R. W., Kim, K. S., & Kim, I. S. (2000). Identification of motifs for cell adhesion within the repeated domains of transforming growth factor- $\beta$ -induced gene,  $\beta$ ig-h3. *Journal of Biological Chemistry*, 275(40), 30907–30915. <https://doi.org/10.1074/jbc.M002752200>
- Kokabu, S., Gamer, L., Cox, K., Lowery, J., Tsuji, K., Raz, R., ... Rosen, V. (2012). BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b. *Molecular Endocrinology (Baltimore, Md.)*, 26(1), 87–94. <https://doi.org/10.1210/me.2011-1168>
- Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., ... Kishimoto, T. (1997). Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, 89(5), 755–764. [https://doi.org/10.1016/S0092-8674\(00\)80258-5](https://doi.org/10.1016/S0092-8674(00)80258-5)
- Komori, T. (2006). Regulation of osteoblast differentiation by transcription factors. *Journal of Cellular Biochemistry*. <https://doi.org/10.1002/jcb.20958>
- Kudo, A. (2017). Introductory review: periostin—gene and protein structure. *Cellular and Molecular Life Sciences*. <https://doi.org/10.1007/s00018-017-2643-5>
- Kudo, A. (2011). Periostin in fibrillogenesis for tissue regeneration: Periostin actions inside and outside the cell. *Cellular and Molecular Life Sciences*. <https://doi.org/10.1007/s00018-011-0784-5>
- Kudo, A., & Kii, I. (2017). Periostin function in communication with extracellular matrices. *Journal of Cell Communication and Signaling*, pp. 1–8. <https://doi.org/10.1007/s12079-017-0422-6>
- Lee, S. J., Lee, E. H., Park, S. Y., & Kim, J. E. (2017). Induction of fibrillin-2 and periostin expression in Osterix-knockdown MC3T3-E1 cells. *Gene*, 596, 123–129. <https://doi.org/10.1016/j.gene.2016.10.018>
- Li, G., Oparil, S., Sanders, J. M., Zhang, L., Dai, M., Chen, L. B., ... Sarembock, I. J. (2006). Phosphatidylinositol-3-kinase signaling mediates vascular smooth muscle cell expression of periostin in vivo and in vitro. *Atherosclerosis*, 188(2), 292–300. <https://doi.org/10.1016/j.atherosclerosis.2005.11.002>
- Li, P., Oparil, S., Feng, W., & Chen, Y.-F. (2004). Hypoxia-responsive growth factors upregulate periostin and osteopontin expression via distinct signaling pathways in rat pulmonary arterial smooth muscle cells. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, 97(4), 1550–8; discussion 1549. <https://doi.org/10.1152/jappphysiol.01311.2003>
- Liebergall, M., Schroeder, J., Mosheiff, R., Gazit, Z., Yoram, Z., Rasooly, L., ... Beyth, S. (2013). Stem cell-based therapy for prevention of delayed fracture union: A randomized and

- prospective preliminary study. *Molecular Therapy*, 21(8), 1631–1638.  
<https://doi.org/10.1038/mt.2013.109>
- Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., ... Feng, X. H. (2006). PPM1A Functions as a Smad Phosphatase to Terminate TGF $\beta$  Signaling. *Cell*, 125(5), 915–928. <https://doi.org/10.1016/j.cell.2006.03.044>
- Lindner, V., Wang, Q., Conley, B. A., Friesel, R. E., & Vary, C. P. H. (2005). Vascular injury induces expression of periostin: Implications for vascular cell differentiation and migration. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(1), 77–83.  
<https://doi.org/10.1161/01.ATV.0000149141.81230.c6>
- Lissenberg-Thunnissen, S. N., De Gorter, D. J. J., Sier, C. F. M., & Schipper, I. B. (2011). Use and efficacy of bone morphogenetic proteins in fracture healing. *International Orthopaedics*. <https://doi.org/10.1007/s00264-011-1301-z>
- Litvin, J., Selim, A. H., Montgomery, M. O., Lehmann, K., Rico, M. C., Devlin, H., ... Safadi, P. F. (2004). Expression and function of periostin-isoforms in bone. *Journal of Cellular Biochemistry*, 92(5), 1044–1061. <https://doi.org/10.1002/jcb.20115>
- Luppen, C. a, Smith, E., Spevak, L., Boskey, A. L., & Frenkel, B. (2003). Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, 18(7), 1186–1197.  
<https://doi.org/10.1359/jbmr.2003.18.7.1186>
- Lyon, T., Scheele, W., Bhandari, M., Koval, K. J., Sanchez, E. G., Christensen, J., ... Huard, F. (2013). Efficacy and safety of recombinant human bone morphogenetic protein-2/calcium phosphate matrix for closed tibial diaphyseal fracture a double-blind randomized controlled phase-ii/iii trial. *Journal of Bone and Joint Surgery - Series A*, 95(23), 2088–2096.  
<https://doi.org/10.2106/JBJS.L.01545>
- Ma, D., Zhang, R., Sun, Y., Rios, H. F., Haruyama, N., Han, X., ... Feng, J. Q. (2011). A novel role of periostin in postnatal tooth formation and mineralization. *Journal of Biological Chemistry*, 286(6), 4302–4309. <https://doi.org/10.1074/jbc.M110.140202>
- Mamalis, A., Markopoulou, C., Lagou, A., & Vrotsos, I. (2011). Oestrogen regulates proliferation, osteoblastic differentiation, collagen synthesis and periostin gene expression in human periodontal ligament cells through oestrogen receptor beta. *Archives of Oral Biology*, 56(5), 446–455. <https://doi.org/10.1016/j.archoralbio.2010.11.001>
- Maruhashi, T., Kii, I., Saito, M., & Kudo, A. (2010). Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase. *Journal of Biological Chemistry*, 285(17), 13294–13303. <https://doi.org/10.1074/jbc.M109.088864>

- Marx, R. E., Armentano, L., Olavarria, A., & Samaniego, J. (2013). rhBMP-2/ACS Grafts Versus Autogenous Cancellous Marrow Grafts in Large Vertical Defects of the Maxilla: An Unsponsored Randomized Open-Label Clinical Trial. *The International Journal of Oral & Maxillofacial Implants*, 28(5), e243–e251. <https://doi.org/10.11607/jomi.te04>
- Matsuzawa, M., Arai, C., Nomura, Y., Murata, T., Yamakoshi, Y., Oida, S., ... Nakamura, Y. (2015). Periostin of human periodontal ligament fibroblasts promotes migration of human mesenchymal stem cell through the  $\alpha\beta3$  integrin/FAK/PI3K/Akt pathway. *Journal of Periodontal Research*, 50(6), 855–863. <https://doi.org/10.1111/jre.12277>
- Merle, B., & Garnero, P. (2012). The multiple facets of periostin in bone metabolism. *Osteoporosis International*. <https://doi.org/10.1007/s00198-011-1892-7>
- Merle, B., Bouet, G., Rousseau, J. C., Bertholon, C., & Garnero, P. (2014). Periostin and transforming growth factor  $\beta$ -induced protein (TGF $\beta$ ip) are both expressed by osteoblasts and osteoclasts. *Cell Biology International*, 38(3), 398–404. <https://doi.org/10.1002/cbin.10219>
- Miyazono, K., Kamiya, Y., & Morikawa, M. (2010). Bone morphogenetic protein receptors and signal transduction. *Journal of Biochemistry*. <https://doi.org/10.1093/jb/mvp148>
- Morris, R. A., Damon, B., Mironov, V., Kasyanov, V., Ramamurthi, A., Moreno-Rodriguez, R., ... Markwald, R. R. (2007). Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. *Journal of Cellular Biochemistry*, 101(3), 695–711. <https://doi.org/10.1002/jcb.21224>
- Nauth, A., Ristiniemi, J., McKee, M. D., & Schemitsch, E. H. (2009). Bone morphogenetic proteins in open fractures: past, present, and future. *Injury*, 40, S27–S31. [https://doi.org/10.1016/S0020-1383\(09\)70008-7](https://doi.org/10.1016/S0020-1383(09)70008-7)
- Nishimura, R., Hata, K., Matsubara, T., Wakabayashi, M., & Yoneda, T. (2012). Regulation of bone and cartilage development by network between BMP signalling and transcription factors. *Journal of Biochemistry*. <https://doi.org/10.1093/jb/mvs004>
- Nishiyama, T., Kii, I., Kashima, T. G., Kikuchi, Y., Ohazama, A., Shimazaki, M., ... Kudo, A. (2011). Delayed re-epithelialization in periostin-deficient mice during cutaneous wound healing. *PLoS ONE*, 6(4). <https://doi.org/10.1371/journal.pone.0018410>
- Nohe, A., Keating, E., Knaus, P., & Petersen, N. O. (2004). Signal transduction of bone morphogenetic protein receptors. *Cellular Signalling*. <https://doi.org/10.1016/j.cellsig.2003.08.011>
- Ogita, M., Rached, M. T., Dworakowski, E., Bilezikian, J. P., & Kousteni, S. (2008). Differentiation and proliferation of periosteal osteoblast progenitors are differentially regulated by estrogens and intermittent parathyroid hormone administration. *Endocrinology*, 149(11), 5713–5723. <https://doi.org/10.1210/en.2008-0369>

- Okamoto, M., Hoshino, T., Kitasato, Y., Sakazaki, Y., Kawayama, T., Fujimoto, K., ... Aizawa, H. (2011). Periostin, a matrix protein, is a novel biomarker for idiopathic interstitial pneumonias. *The European Respiratory Journal*, *37*(5), 1119–27. <https://doi.org/10.1183/09031936.00059810>
- Onichtchouk, D., Chen, Y. G., Dosch, R., Gawantka, V., Delius, H., Massagué, J., & Niehrs, C. (1999). Silencing of TGF- $\beta$  signalling by the pseudoreceptor BAMBI. *Nature*, *401*(6752), 480–485. <https://doi.org/10.1038/46794>
- Ontsuka, K., Kotobuki, Y., Shiraishi, H., Serada, S., Ohta, S., Tanemura, A., ... Naka, T. (2012). Periostin, a matricellular protein, accelerates cutaneous wound repair by activating dermal fibroblasts. *Experimental Dermatology*, *21*(5), 331–336. <https://doi.org/10.1111/j.1600-0625.2012.01454.x>
- Oryan, A., Alidadi, S., Moshiri, A., & Bigham-Sadegh, A. (2014). Bone morphogenetic proteins: A powerful osteoinductive compound with non-negligible side effects and limitations. *BioFactors*. <https://doi.org/10.1002/biof.1177>
- Oshima, A., Tanabe, H., Yan, T., Lowe, G. N., Glackin, C. A., & Kudo, A. (2002). A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, Twist. *Journal of Cellular Biochemistry*, *86*(4), 792–804. <https://doi.org/10.1002/jcb.10272>
- Otto, F., Kanegane, H., & Mundlos, S. (2002). Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. *Human Mutation*, *19*(3), 209–216. <https://doi.org/10.1002/humu.10043>
- Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., ... Owen, M. J. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, *89*(5), 765–771. [https://doi.org/10.1016/S0092-8674\(00\)80259-7](https://doi.org/10.1016/S0092-8674(00)80259-7)
- Ouyang, G., Liu, M., Ruan, K., Song, G., Mao, Y., & Bao, S. (2009). Upregulated expression of periostin by hypoxia in non-small-cell lung cancer cells promotes cell survival via the Akt/PKB pathway. *Cancer Letters*, *281*(2), 213–219. <https://doi.org/10.1016/j.canlet.2009.02.030>
- Padial-Molina, M., Marchesan, J. T., Taut, A. D., Jin, Q., Giannobile, W. V., & Rios, H. F. (2012). Methods to validate tooth-supporting regenerative therapies. *Methods in Molecular Biology*, *887*, 135–148. [https://doi.org/10.1007/978-1-61779-860-3\\_13](https://doi.org/10.1007/978-1-61779-860-3_13)
- Papakostidis, C., Kontakis, G., Bhandari, M., & Giannoudis, P. V. (2008). Efficacy of autologous iliac crest bone graft and bone morphogenetic proteins for posterolateral fusion of lumbar spine: A meta-analysis of the results. *Spine*, *33*(19). <https://doi.org/10.1097/BRS.0b013e3181844eca>

- Riggins, G. J., Thiagalingam, S., Rozenblum, E., Weinstein, C. L., Kern, S. E., Hamilton, S. R., ... Vogelstein, B. (1996). Mad-related genes in the human. *Nature Genetics*, 13(3), 347–349. <https://doi.org/10.1038/ng0796-347>
- Rios, H., Koushik, S. V., Wang, H., Wang, J., Zhou, H.-M., Lindsley, A., ... Conway, S. J. (2005). periostin Null Mice Exhibit Dwarfism, Incisor Enamel Defects, and an Early-Onset Periodontal Disease-Like Phenotype. *Molecular and Cellular Biology*, 25(24), 11131–11144. <https://doi.org/10.1128/MCB.25.24.11131-11144.2005>
- Rios, H. F., Ma, D., Xie, Y., Giannobile, W. V., Bonewald, L. F., Conway, S. J., & Feng, J. Q. (2008). Periostin Is Essential for the Integrity and Function of the Periodontal Ligament During Occlusal Loading in Mice. *Journal of Periodontology*, 79(8), 1480–1490. <https://doi.org/10.1902/jop.2008.070624>
- Ristinieniemi, J., Flinkkilä, T., Hyvönen, P., Lakovaara, M., Pakarinen, H., & Jalovaara, P. (2007). RhBMP-7 accelerates the healing in distal tibial fractures treated by external fixation. *The Journal of Bone and Joint Surgery. British Volume*, 89(2), 265–72. <https://doi.org/10.1302/0301-620X.89B2.18230>
- Roh, J. S., Yeung, C. A., Field, J. S., & McClellan, R. T. (2013). Allogeneic morphogenetic protein vs. Recombinant human bone morphogenetic protein-2 in lumbar interbody fusion procedures: A radiographic and economic analysis. *Journal of Orthopaedic Surgery and Research*, 8(1). <https://doi.org/10.1186/1749-799X-8-49>
- Romanos, G. E., Asnani, K. P., Hingorani, D., & Deshmukh, V. L. (2014). PERIOSTIN: Role in Formation and Maintenance of Dental Tissues. *Journal of Cellular Physiology*, 229(1), 1–5. <https://doi.org/10.1002/jcp.24407>
- Rosenzweig, B. L., Imamura, T., Okadome, T., Cox, G. N., Yamashita, H., ten Dijke, P., ... Miyazono, K. (1995). Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 92(17), 7632–6. <https://doi.org/10.1073/pnas.92.17.7632>
- Ruan, K., Bao, S., & Ouyang, G. (2009). The multifaceted role of periostin in tumorigenesis. *Cellular and Molecular Life Sciences*. <https://doi.org/10.1007/s00018-009-0013-7>
- Sasaki, H., Yu, C.-Y., Dai, M., Tam, C., Loda, M., Auclair, D., ... Elias, A. (2003). Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer. *Breast Cancer Research and Treatment*, 77(3), 245–52. <https://doi.org/10.1023/A:1021899904332>
- Seidler, N. W. (2013). Basic biology of GAPDH. *Advances in Experimental Medicine and Biology*, 985, 1–36. <https://doi.org/10.1007/978-94-007-4716-6-1>



- Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahim, J., ... Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *The Lancet*, *364*(9429), 149–155. [https://doi.org/10.1016/S0140-6736\(04\)16627-0](https://doi.org/10.1016/S0140-6736(04)16627-0)
- Shao, R., Bao, S., Bai, X., Blanchette, C., Anderson, R. M., Dang, T., ... Wang, X. (2004). Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. *Molecular and Cellular Biology*, *24*(9), 3992–4003. <https://doi.org/10.1128/MCB.24.9.3992>
- Shi, S., de Gorter, D. J. J., Hoogaars, W. M. H., 't Hoen, P. A. C., & ten Dijke, P. (2013). Overactive bone morphogenetic protein signaling in heterotopic ossification and Duchenne muscular dystrophy. *Cellular and Molecular Life Sciences : CMLS*, *70*(3), 407–23. <https://doi.org/10.1007/s00018-012-1054-x>
- Shi, W., Chang, C., Nie, S., Xie, S., Wan, M., & Cao, X. (2007). Endofin acts as a Smad anchor for receptor activation in BMP signaling. *Journal of Cell Science*, *120*(7), 1216–1224. <https://doi.org/10.1242/jcs.03400>
- Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., & Cao, X. (2004). GADD34-PP1c recruited by Smad7 dephosphorylates TGF $\beta$  type I receptor. *Journal of Cell Biology*, *164*(2), 291–300. <https://doi.org/10.1083/jcb.200307151>
- Shimazaki, M., Nakamura, K., Kii, I., Kashima, T., Amizuka, N., Li, M., ... Kudo, A. (2008). Periostin is essential for cardiac healing after acute myocardial infarction. *The Journal of Experimental Medicine*, *205*(2), 295–303. <https://doi.org/10.1084/jem.20071297>
- Siriwardena, B. S. M. S., Kudo, Y., Ogawa, I., Kitagawa, M., Kitajima, S., Hatano, H., ... Takata, T. (2006). Periostin is frequently overexpressed and enhances invasion and angiogenesis in oral cancer. *British Journal of Cancer*, *95*(10), 1396–1403. <https://doi.org/10.1038/sj.bjc.6603431>
- Sodek, J. (1977). A comparison of the rates of synthesis and turnover of collagen and non-collagen proteins in adult rat periodontal tissues and skin using a microassay. *Archives of Oral Biology*, *22*(12), 655–665. [https://doi.org/10.1016/0003-9969\(77\)90095-4](https://doi.org/10.1016/0003-9969(77)90095-4)
- Stewart, S., Gomez, A. W., Armstrong, B. E., Henner, A., & Stankunas, K. (2014). Sequential and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Reports*, *6*(3), 482–498. <https://doi.org/10.1016/j.celrep.2014.01.010>
- Stock, M., Schäfer, H., Fliegau, M., & Otto, F. (2004). Identification of novel target genes of the bone-specific transcription factor Runx2. *Journal of Bone and Mineral Research*, *19*(6), 959–972. <https://doi.org/10.1359/jbmr.2004.19.6.959>
- Sugiura, T., Takamatsu, H., Kudo, A., & Amann, E. (1995). Expression and characterization of murine osteoblast-specific factor 2 (OSF-2) in a baculovirus expression system. *Protein Expression and Purification*, *6*(3), 305–11. <https://doi.org/10.1006/prev.1995.1040>

- Suzuki, C., Murakami, G., Fukuchi, M., Shimanuki, T., Shikauchi, Y., Imamura, T., & Miyazono, K. (2002). Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *Journal of Biological Chemistry*, 277(42), 39919–39925. <https://doi.org/10.1074/jbc.M201901200>
- Suzuki, H., Amizuka, N., Kii, I., Kawano, Y., Nozawa-Inoue, K., Suzuki, A., ... Maeda, T. (2004). Immunohistochemical localization of periostin in tooth and its surrounding tissues in mouse mandibles during development. *Anatomical Record - Part A Discoveries in Molecular, Cellular, and Evolutionary Biology*, 281(2), 1264–1275. <https://doi.org/10.1002/ar.a.20080>
- Swiontkowski, M. F., Aro, H. T., Donell, S., Esterhai, J. L., Goulet, J., Jones, A., ... Patel, A. (2006). Recombinant human bone morphogenetic protein-2 in open tibial fractures. A subgroup analysis of data combined from two prospective randomized studies. *The Journal of Bone and Joint Surgery. American Volume*, 88(6), 1258–1265. <https://doi.org/10.2106/JBJS.E.00499>
- Takayama, G., Arima, K., Kanaji, T., Toda, S., Tanaka, H., Shoji, S., ... Izuhara, K. (2006). Periostin: A novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *Journal of Allergy and Clinical Immunology*, 118(1), 98–104. <https://doi.org/10.1016/j.jaci.2006.02.046>
- Takayama, I., & Kudo, A. (2012). Periostin in dental science. *Japanese Dental Science Review*. <https://doi.org/10.1016/j.jdsr.2012.02.001>
- Takeshita, S., Kikuno, R., Tezuka, K., Amann, E., Wu, Y., Yin, J., ... Desrosiers, M. (1993). Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. *Biochemical Journal*, 294(1), 271–278. <https://doi.org/10.1042/bj2940271>
- Tanabe, H., Takayama, I., Nishiyama, T., Shimazaki, M., Kii, I., Li, M., ... Kudo, A. (2010). Periostin associates with notch1 precursor to maintain notch1 expression under a stress condition in mouse cells. *PLoS ONE*, 5(8). <https://doi.org/10.1371/journal.pone.0012234>
- Tanaka, Y., Nakayamada, S., & Okada, Y. (2005). Osteoblasts and Osteoclasts in Bone Remodeling and Inflammation. *Current Drug Target -Inflammation & Allergy*, 4(3), 325–328. <https://doi.org/10.2174/1568010054022015>
- Tilman, G., Mattiussi, M., Brasseur, F., van Baren, N., & Decottignies, A. (2007). Human periostin gene expression in normal tissues, tumors and melanoma: Evidences for periostin production by both stromal and melanoma cells. *Molecular Cancer*, 6. <https://doi.org/10.1186/1476-4598-6-80>
- Tsiridis, E., Upadhyay, N., & Giannoudis, P. (2007). Molecular aspects of fracture healing: which are the important molecules? *Injury*, 38 Suppl 1, S11-25. [https://doi.org/S0020-1383\(07\)00055-1](https://doi.org/S0020-1383(07)00055-1) [pii] <https://doi.org/10.1016/j.injury.2007.02.006>

- Urist, M. R., Mikulski, A., & Lietze, A. (1979). Solubilized and insolubilized bone morphogenetic protein. *Proceedings of the National Academy of Sciences of the United States of America*, 76(4), 1828–32. <https://doi.org/10.1073/pnas.76.4.1828>
- Urist, M. R. (1965). Bone: Formation by Autoinduction(Citation). *Science*, 150(3698), 893–899. Retrieved from <http://science.sciencemag.org/content/150/3698/893.abstract>
- Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., & Franceschi, R. T. (1999). Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. *Journal of Bone and Mineral Research*, 14(6), 893–903. <https://doi.org/10.1359/jbmr.1999.14.6.893>
- Watanabe, T., Yasue, A., Fujihara, S., & Tanaka, E. (2012). PERIOSTIN regulates MMP-2 expression via the  $\alpha\beta3$  integrin/ERK pathway in human periodontal ligament cells. *Archives of Oral Biology*, 57(1), 52–59. <https://doi.org/10.1016/j.archoralbio.2011.07.010>
- Wen, W., Chau, E., Jackson-Boeters, L., Elliott, C., Daley, T. D., & Hamilton, D. W. (2010). TGF- $\beta$ 1 and FAK regulate periostin expression in PDL fibroblasts. *Journal of Dental Research*, 89(12), 1439–1443. <https://doi.org/10.1177/0022034510378684>
- Wilde, J., Yokozeki, M., Terai, K., Kudo, A., & Moriyama, K. (2003). The divergent expression of periostin mRNA in the periodontal ligament during experimental tooth movement. *Cell and Tissue Research*, 312(3), 345–351. <https://doi.org/10.1007/s00441-002-0664-2>
- Wilkinson, L., Kollé, G., Wen, D., Piper, M., Scott, J., & Little, M. (2003). CRIM1 Regulates the Rate of Processing and Delivery of Bone Morphogenetic Proteins to the Cell Surface. *Journal of Biological Chemistry*, 278(36), 34181–34188. <https://doi.org/10.1074/jbc.M301247200>
- Winnier, G., Blessing, M., Labosky, P. A., & Hogan, B. L. M. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes and Development*, 9(17), 2105–2116. <https://doi.org/10.1101/gad.9.17.2105>
- Woodruff, P. G., Boushey, H. a, Dolganov, G. M., Barker, C. S., Yang, Y. H., Donnelly, S., ... Fahy, J. V. (2007). Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proceedings of the National Academy of Sciences of the United States of America*, 104(40), 15858–63. <https://doi.org/10.1073/pnas.0707413104>
- Woodruff, P. G., Modrek, B., Choy, D. F., Jia, G., Abbas, A. R., Ellwanger, A., ... Fahy, J. V. (2009). T-helper type 2-driven inflammation defines major subphenotypes of asthma. *American Journal of Respiratory and Critical Care Medicine*, 180(5), 388–395. <https://doi.org/10.1164/rccm.200903-0392OC>

- Wozney, J. M., Rosen, V., Celeste, a J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., ... Wang, E. a. (1988). Novel regulators of bone formation: molecular clones and activities. *Science*, 242, 1528–1534. <https://doi.org/10.1126/science.3201241>
- Yamada, S., Tauchi, T., Awata, T., Maeda, K., Kajikawa, T., Yanagita, M., & Murakami, S. (2014). Characterization of a novel periodontal ligament-specific periostin isoform. *Journal of Dental Research*, 93(9), 891–897. <https://doi.org/10.1177/0022034514543015>
- Zhang, H., & Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development (Cambridge, England)*, 122(10), 2977–2986. <https://doi.org/10.1016/J.YDBIO.2004.12.037>
- Zhu, S., Barbe, M. F., Liu, C., Hadjiargyrou, M., Popoff, S. N., Rani, S., ... Litvin, J. (2009). Periostin-like-factor in osteogenesis. *Journal of Cellular Physiology*, 218(3), 584–592. <https://doi.org/10.1002/jcp.21633>
- Zhu, W., Kim, J., Cheng, C., Rawlins, B. A., Boachie-Adjei, O., Crystal, R. G., & Hidaka, C. (2006). Noggin regulation of bone morphogenetic protein (BMP) 2/7 heterodimer activity in vitro. *Bone*, 39(1), 61–71. <https://doi.org/10.1016/j.bone.2005.12.018>
- Zhu, W., Rawlins, B. a, Boachie-Adjei, O., Myers, E. R., Arimizu, J., Choi, E., ... Hidaka, C. (2004). Combined bone morphogenetic protein-2 and -7 gene transfer enhances osteoblastic differentiation and spine fusion in a rodent model. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, 19(12), 2021–2032. <https://doi.org/10.1359/JBMR.040821>

## Curriculum Vitae

**VINCENT KHANG**

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### ACADEMIC INFORMATION

**Masters in Oral Biology (MSc), Certificate in Orthodontics** **Anticipated: May 2018**  
University of Nevada, Las Vegas, *Las Vegas, NV USA*

**Doctor of Dental Surgery (DDS)** **2015**  
New York University College of Dentistry, *New York, NY USA*

**Program for Survivors of Torture, Honors Program (Fourth Year Elective)** **2014-2015**

New York University College of Dentistry, *New York, NY USA*  
Providing comprehensive dental treatment to survivors of torture who have immigrated to the USA. All care is financially sponsored by the program.

**Bachelor of Science, Human Biology & Biochemistry Double Major, Psychology Minor** **2010**  
University of Toronto, *Toronto, ON Canada*

### RESEARCH EXPERIENCE

**Masters Research Thesis** **2015-2018**  
Dr. Brian Chrzan, Dept. of Orthodontics of University of Nevada, Las Vegas, *Las Vegas, NV USA*

Examining the effect of various cytokines on the expression of pre-osteoblastic markers in a mouse cell line.

**Research Student** **2014-2015**  
Dr. Mitchell Lipp, Dept. of Orthodontics of New York University College of Dentistry, *New York, NY USA*

Integrating course material into the Articulate Storyline software and examining the effects on student learning of a third year orthodontics seminar course.

**Research Student** **2009-2010**  
Dr. Joanne Rovet, Faculty of Psychology of the University of Toronto, *Toronto, ON Canada*  
Examining the role of gender on the visual perceptual pathways of the brain through execution and analysis of participant testing.

## LEADERSHIP SKILLS

### Volunteer Dentist

May 2016

Give Kids A Smile of University of Nevada Las Vegas, *Las Vegas, NV USA*  
Conducting orthodontic screenings and overseeing dental care to pediatric patients.

### Outreach Member

April 2015

New York University College of Dentistry Global Outreach, *Machias, ME USA*  
Providing dental care to patients with limited access to care in remote Maine.

### Outreach Member

July 2013 – May 2014

Dr. Andrew Schenkel of New York University College of Dentistry, *New York, NY USA*  
Providing dental screenings and oral hygiene instructions to participants at community health fairs throughout NYC.

### Outreach Member

Feb – April 2012

Dr. Ellen Lee of New York University College of Dentistry, *New York, NY USA*  
Conducting oral hygiene presentations and distributing oral hygiene products to Chinese seniors at nursing homes.

## TEACHING EXPERIENCE

### Clinical Tutor

October 2013

ADEA Lab Tutorial of New York University College of Dentistry, *New York, NY USA*  
Assisting second year dental students with crown and bridge typodont preparations.

## AWARDS

### Omicron Kappa Upsilon (OKU) Inductee

2015

Dental Honors Society

### Dr. Elsa Pleuger Rahmsdorff Scholarship

2013

New York University College of Dentistry scholarship for academic excellence in first year dental school.

## OTHER SKILLS

Fluent in Cantonese language.