**University of Iceland**

**Faculty of Medicine**

**31.05.2011**

# Role of Periostin/Periostin-Like-Factor in BMP-2 induced osteoblastic differentiation

**Bára Dís Benediktsdóttir**

**Supervisors: Dr. Chafik Ghayor, Dr. Franz E. Weber**

**University hospital of Zürich**
**Oral Biotechnology & Bioengineering Department**
**Cranio-Maxillofacial Surgery**
**Freuklinikstrasse 24 (Nord2 B-843)**
**8091 Zürich**
**Switzerland**

**Key words: Periostin, Periostin-Like-Factor, BMP-2, TNF-α, osteoblastic differentiation**

**Role of Periostin/Periostin-Like-Factor in BMP-2 induced osteoblastic differentiation**

**University hospital of Zürich**
**Oral Biotechnology & Bioengineering Department**
**Cranio-Maxillofacial Surgery**
**Freuklinikstrasse 24 (Nord2 B-843)**
**8091 Zürich**
**Switzerland**

# Table of Contents

# 1. Abstract


## 1.1. Introduction

Periostin is a 90 kDa protein which is predominantly secreted by Mesenchymal stem cells (MSCs) in the bone marrow stroma and by osteoblasts. Periostin-Like-Factor (PLF) is a isoform of Periostin and these isoforms are highly homologous because they are alternatively spliced mRNAs from the same gene. Periostin/PLF promotes osteoblastic differentiation in vitro and bone formation in vivo. BMPs are growth factors that are known to induce bone formation and BMP-2 is one of the most potent growth factor that stimulates osteoblastic differentiation. TNF-α is an inflammatory cytokine that stimulates osteoclastic differentiation while simultaneously inhibiting BMP-2 induced osteoblastic differentiation. The aim of this study is divided into two parts: 1) To investigate whether there is collaboration between BMP-2 and Periostin/PLF during osteoblastic differentiation, 2) To investigate whether there is a correlation between expression level of Periostin/PLF and the inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

## 1.2. Materials and Methods

Experiments were done in both C2C12 cells (pluripotent stem cells) and MC3T3-E1 cells (pre-osteoblasts). Cells were transfected with expression plasmid or siRNA of Periostin/PLF and then treated with BMP-2 and/or TNF-α. After treatment, alkaline phosphatase (ALP) was measured as a marker of osteoblastic differentiation. The amount of Periostin/PLF mRNA and protein expression were determined by real time RT-qPCR and Western blot analysis respectively.

## 1.3. Results

Periostin/PLF protein expression is increased by BMP-2 and decreased by TNF-α in both MC3T3-E1 and C2C12 cells. Transient transfection with periostin/PLF plasmid (over-expression) substantially increased BMP-2 stimulated ALP activity ($p<0.05$) and protein level compared to non-transfected cells. Furthermore, transient transfection with Periostin/PLF-siRNA (silencing) substantially reduced BMP-2 stimulated ALP activity ($p<0.05$) and protein level compared to non-transfected cells. Finally, Periostin/PLF over-expression or silencing with siRNA did not restore the ALP activity in cells treated with BMP-2 and TNF-α.

## 1.4. Conclusion

Periostin/PLF potentiates the effect of BMP-2 in osteoblastic differentiation and BMP-2 increases protein expression of Periostin/PLF. Although treatment with TNF-α resulted in lower amount of Periostin/PLF protein expressed, no changes in ALP activity were recorded in transfected cells treated with BMP-2 and TNF-α. Therefore, Periostin/PLF is not involved in the inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

# 2. Abbreviations

Follow is a list of abbreviations in alphabetical order:

**aa:** Amino acids

**ALP:** Alkaline phosphatase

**Alpha-MEM:** Alpha modified Eagle's medium

**ANOVA:** Analysis of variance

**BMPs:** Bone morphogenetic proteins

**BMP-2:** Bone morphogenetic protein 2

**bp:** Base pairs

**BSA:** Bovine serum albumin

**C2C12:** Pluripotent cells. Mouse cell line that is able to differentiate in osteoblasts when treated with BMP2.

**D-MEM:** Dulbecco's modified Eagle's medium

**DPBS IX:** Dulbecco's Phosphate Buffered Saline

***E. coli:*** *Escherichia coli*

**F:** Forward (refers to primers for RT-qPCR)

**FBS:** Fetal Bovine serum

**FDA:** US Food and Drug Administration

**GAPDH:** Glyceraldehyde-3-phosphate-dehydrogenase

**IPTG:** Isopropy-1-thio-β-D-galactopyranoside

**kDa:** Kilodalton

**MC3T3-E1:** Mouse calvaria-derived osteoblast-like cell line

**MSCs:** Mesenchymal Stem Cells

**PI3-K/Akt:** Phosphatidylinositol 3-kinases/Protein kinase B

**PLF:** Periostin-Like-Factor

**PVDF:** Polyvinylidene difluoride

**R:** Reverse (refers to primers for RT-qPCR)

**Real time RT-qPCR:** Real time reverse transcriptase quantitative polymerase chain reaction

**rh-BMP-2:** Recombinant human BMP-2

**RIPA-buffer:** Radioimmunoprecipitation assay buffer

**Runx2:** Runt related transcription factor 2

**PTH:** Parathyroid hormone

**SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis

**SDS:** Sodium dodecyl sulfate

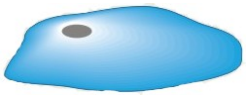
**siRNA:** Small interfering RNA

**TBST:** Tris-Buffered Saline Tween

**TGF-β:** Transforming growth factor beta

**TNF-α:** Tumor necrosis factor-alpha

## 3. Key to figures



**MSC**

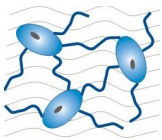
**Lining cell**

**Pre-osteoblast**

**Pre-osteoclast**

**Osteoblast**

**Osteoclast**



**Osteocytes**

# 4. Introduction

The bones in the skeleton composite of three main cell types, osteoblasts, osteocytes and osteoclasts. Osteoblasts are derived from the Mesenchymal Stem Cells (MSCs) of the bone marrow stroma. Under the right condition MSCs cells change to pre-osteoblast cells, and finally to mature osteoblasts. This process is called osteoblastic differentiation (see Fig. 1). Osteoblastic differentiation can be determined by measuring Alkaline phosphatase (ALP), which is a marker of osteoblastic differentiation, or by measuring mRNA expression of Runt related transcription factor 2 (Runx2), which is a master gene of osteoblastic differentiation.[1]

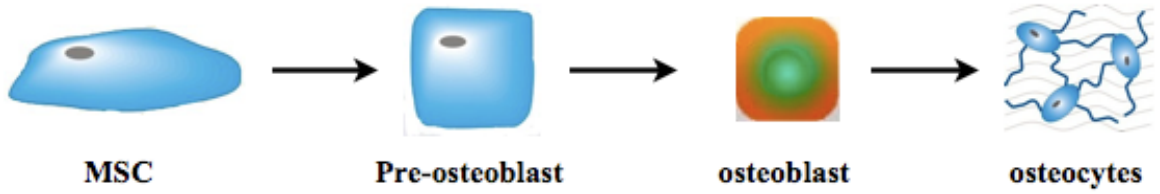



**Figure 1:** A schematic figure of osteoblastic differentiation. Osteoblasts originate from MSCs which are also capable to differentiate into other cell types, e.g. muscle, fat and ligament cells. During bone formation some of the osteoblasts become entrapped in the newly formed bone matrix and are then called osteocytes. Osteocytes are the terminal stage of osteoblastic differentiation.[5]

Osteoblasts synthesize the collagen bone matrix and mediate its mineralization, and are therefore responsible for bone formation.[2,3] Some of the osteoblasts become entrapped within the matrix as it is being synthesized, and are then called osteocytes. Others remain at the surface as thin cells and are called lining cells. Osteocytes are the most numerous cells in the bone and are widely connected to the osteoblasts at the surface. Osteoclasts are giant, multinucleate cells that remove bone by breaking down the matrix in a process called bone resorption.[3-5]

The skeleton serves both as a structural function, providing support and mobility, and as a reservoir function for the essential minerals, calcium and phosphorus. These two different functions are in competition with each other, which can make it difficult to maintain bone health. The bones need to be strong and able to repair any damage and that requires plentiful stores of calcium and phosphorus. When there is a deficiency of these supplies in the body the regulating hormones, e.g. Parathyroid hormone (PTH), take them out of the bones to serve essential functions in other organs. To respond to this dual role of the skeleton, bones are constantly changing in size and shape during life. The adult skeleton is mostly replaced every ten years.[5]

There are two processes that modulate these changes, bone modeling and bone remodeling.

Bone modeling is the process by which new bone is formed at one site and old bone is removed from another site of the same bone (see Fig. 2). This process is vigorous during childhood and allows bones to change in size and shape.

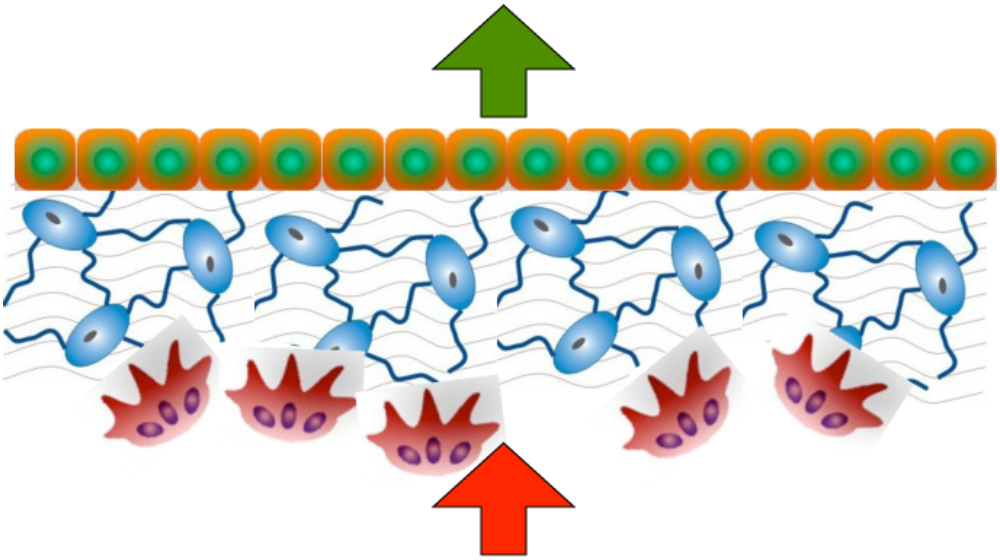


**Figure 2:** In bone modeling the action of osteoblasts and osteoclasts are not linked. Osteoblasts synthesize new bone matrix (green arrow) at one site of the bone and osteoclasts resorb bone (red arrow) at another location within the same bone. By this process rapid changes can occur in the amount and shape of bone.[5]

Bone remodeling is a physiological process that occurs throughout life and becomes the dominant process in early adulthood. In bone remodeling, small amount of bone is first absorbed by osteoclasts and then new bone is formed at the same site by osteoblasts (see Fig. 3).

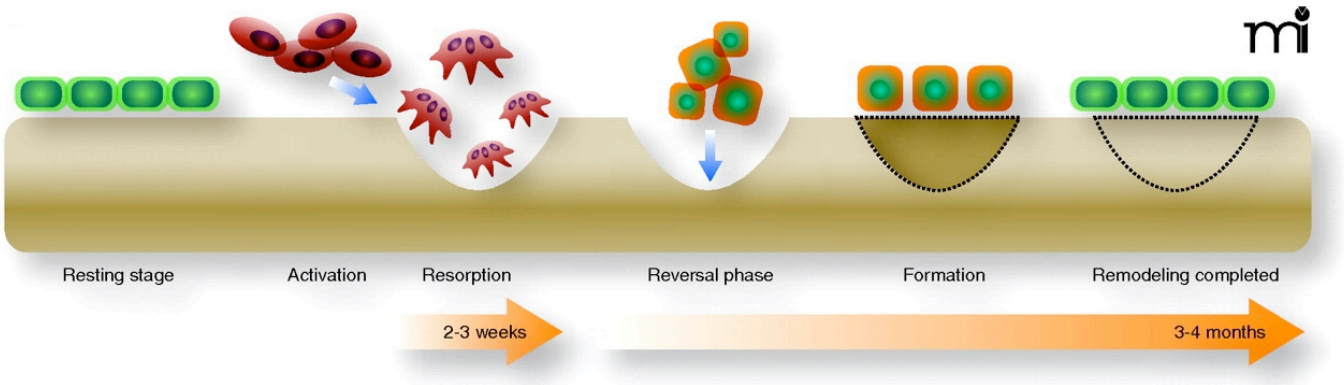



**Figure 3:** Normal bone remodeling consist of activation, bone resorption, reversal phase and bone formation. In the activation step osteoclast precursor cells become osteoclasts. Osteoclasts then start to remove the old bone, schematized here as wells in the bone. A brief reversal phase follows which the resorbed surface is prepared for further bone formation. Finally the osteoblasts starts to synthesize new bone matrix. The resorption phase is relatively quick, lasting for 2-3 weeks, while the latter formation phase can take several months to complete.[5,7] *Orginal figure from: Drake 2010. http://molinterv.aspetjournals.org/content/10/3/141/F2.large.jpg*

Bone remodeling is vital for maintaining bone health and a dynamic skeleton. The main purpose of bone remodeling during adulthood is to maintain bone strength by removing brittle old bone and by that prevent its accumulation. Bone resorption is important in metabolic perspective because the bones can supply needed calcium and phosphorus, e.g. during pregnancy or when there is a deficiency in the diet. Bone resorption is not damaging for bones unless it turn excessive. In normal bone remodeling, the balance between resorption by osteoclasts and formation by osteoblasts is regulated by systemic hormones and local factors, such as growth factors and cytokines.[3-7] The loss of this fine balance is the primary cause of bone diseases like osteoporosis and osteopetrosis (see Fig. 4). Therefore, it is of scientific and clinical importance to understand this process and study the factors that regulate bone formation and bone resorption.[5]

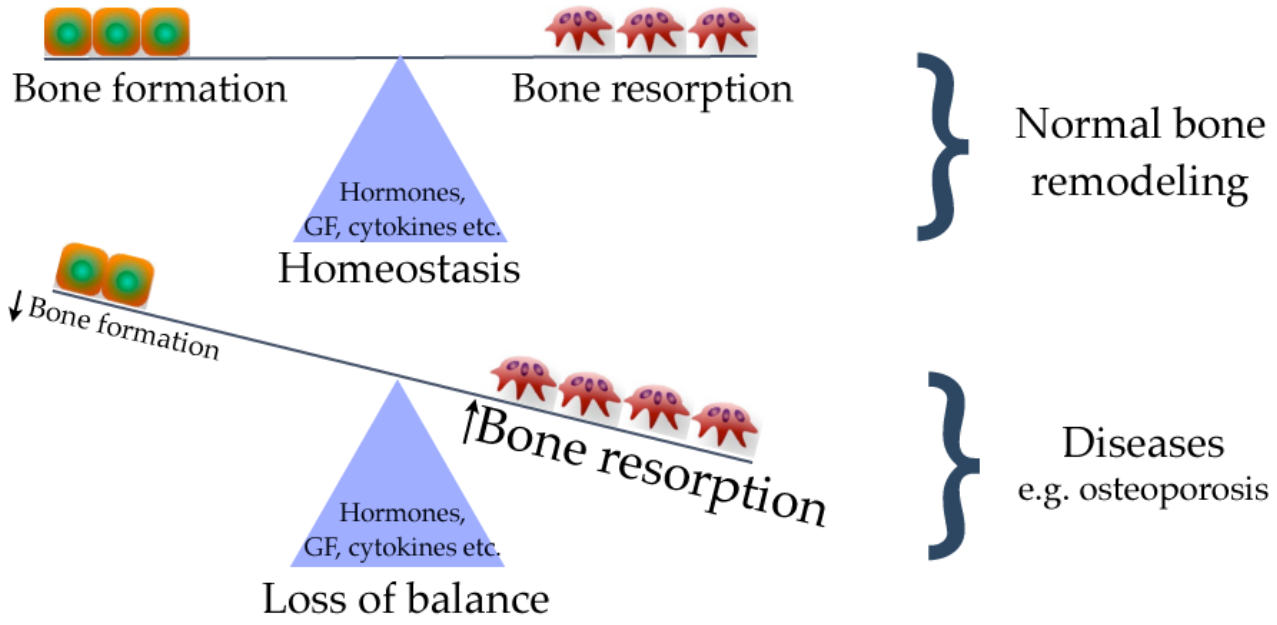



**Figure 4:** A schematic figure of the balance between bone formation and bone resorption which needs to be maintained in order to keep bones healthy. Net changes in the amount and shape of bone are minimal in normal bone remodeling. With loss of balance changes in the amount and shape will occur.[5]

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that are members of the transforming growth factor β (TGF-β) superfamily and exert a wide range of biological effects in different tissues. BMP signaling is critical for heart, neural and cartilage development and for postnatal bone formation. In particular BMPs contribute to the formation of bone by inducing differentiation of Mesenchymal Stem Cells (MSCs) to bone-forming cells, such as osteoblasts. In bone tissue engineering, BMPs are the most potent factors for inducing bone formation. Around

twenty BMP family members have been identified.[8] BMP-2 expression has been identified in a large variety of cells including osteoblasts (see Fig. 5). BMP-2 is one of the most potent growth

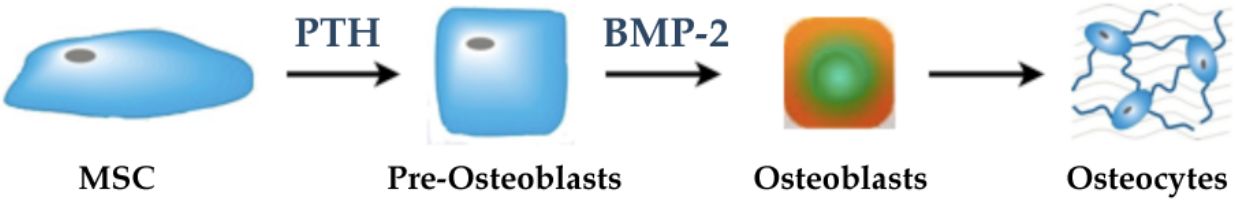



**Figure 5:** PTH increases differentiation from MSCs. BMPs stimulates the differentiation along osteoblast direction. *Orginal picture from: Nanes M.S. 2003.*[15]

factor that stimulates osteoblastic differentiation and bone formation.[9,10] Recombinant human BMP-2 (rh-BMP-2, INFUSE® Bone Graft) is used in clinic for various indications related to bone repair, e.g. in orthopedics to induce migration, proliferation and differentiation of MSCs into osteoblasts.[11-13] rhBMP-2 is combined with a scaffold (with an absorbable collagen sponge) that permits its retention and release at the wound site in the bone.[13,14] rhBMP-2 has been approved by the US Food and Drug Administration (FDA) as a replacement or an alternative to autogenous bone grafting in certain clinical indications, e.g. in fusion of spinal vertebrae and in long bone fractures.[11,14]

Tumor necrosis factor alpha (TNF-α) is a inflammatory cytokine that stimulates osteoclastic differentiation while simultaneously inhibiting bone formation action of osteoblasts. This can alter the balance between bone formation and bone resorption. It has been shown that TNF-α blocks osteoblastic differentiation in MC3T3-E1 (pre-osteoblasts) and C2C12 cells (pluripotent cells) by inhibiting BMP-2 induced osteoblastic differentiation. By this manner, TNF-α suppresses recruitment of osteoblasts from MSCs and pre-osteoblasts.[15-17]

Periostin was first identified in MC3T3-E1 pre-osteoblasts cells and was thought at that time to be bone-specific.[18] Periostin is a disulfide linked protein, 811 amino acid (aa) and about 90 kilodalton (kDa). Periostin protein contains a typical N-terminal secretory signal sequence but lacks a typical transmembrane domain, followed by four internal homologous repeat regions which precede the C-terminal domain.[19,20] Periostin belongs to a family of vitamin K-dependent gamma carboxylated proteins, characterized by the presence of fasciclin domains. Periostin is expressed and secreted by MSCs in the bone marrow stroma, which have the capacity to differentiate into many mesenchymal cell lineages, including osteoblasts.[21] Furthermore, is Periostin secreted by osteoblasts and osteoblast-like cell lines.[20] Periostin is predominantly expressed in periosteum,

periodontal ligaments, tendons, heart valves and cornea.[22] Its function during skeletal development and its precise location in the bone matrix are however unclear.[23]

Periostin has also been shown to be involved in the development of heart and other tissues. Periostin is re-expressed in adults e.g. after myocardial injuries[24,25] and bone fracture,[26] and recently it was reported that Periostin is a therapeutic target for heart disease.[27] Periostin is over-expressed in various types of malignant tumors and correlates with disease progression and the aggressiveness of the tumors. High stromal or epithelium expression of Periostin is associated with poor survival of patients.[28-33] Periostin interacts with many types of cell-surface receptor, especially integrins, and signals notably via the PI3-K/Akt pathway to promote cancer cell survival, invasion, angiogenesis and metastasis in several types of cancer.[29,31,34-36] Furthermore serum levels of Periostin are often increased in such cases, e.g. in breast cancer bone metastasis. This suggest that Periostin could be a biochemical marker of bone metastasis formation. Its role in bone metastasis is however unclear.[34]

An isoform of Periostin has been identified and is referred to as Periostin-like-factor (PLF). Periostin isoforms are expressed in vivo and in vitro during osteoblastic differentiation. Periostin and PLF are highly homologous because they arise from the same gene and alternatively spliced mRNAs. The major difference between PLF and Periostin is the C-terminal sequence. PLF and Periostin are different in two specific regions, between 673 and 699 aa and 785-812 aa (see Fig. 6). Thus PLF contains a sequence of 27 aa (673-699 aa, comprise exon 17) that is absent in Periostin and there is a 28 aa (785-812 aa, comprise exon 21) sequence in Periostin that is not found in PLF (see Fig. 7). Their mRNA are present in pre-osteoblasts cells. Some hypothesize that these isoforms may have significant functional consequences, but it has not been examined in details.[20] Periostin/ PLF is both produced and secreted by osteoblasts in vitro. It was recently reported that Periostin/ PLF promotes and accelerates osteoblastic proliferation and differentiation in vitro, and promotes bone formation in vivo.[16,37]

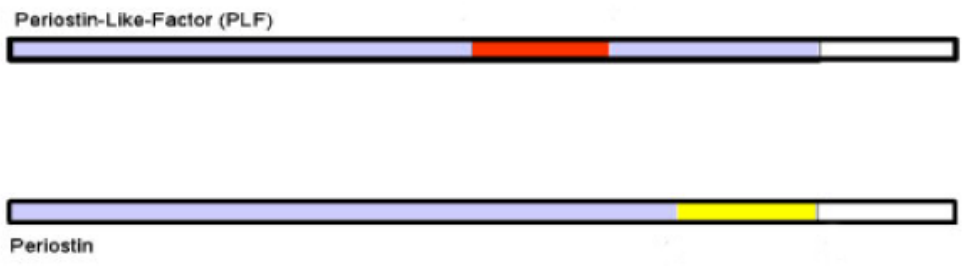



**Figure 6:** The figure demonstrates the aa sequence of PLF and Periostin. The red (673-699 aa) and yellow (785-812 aa) boxes represent the aa sequences that differ in these two isoforms. [20] *Figure from Litvin et al. 2004.*

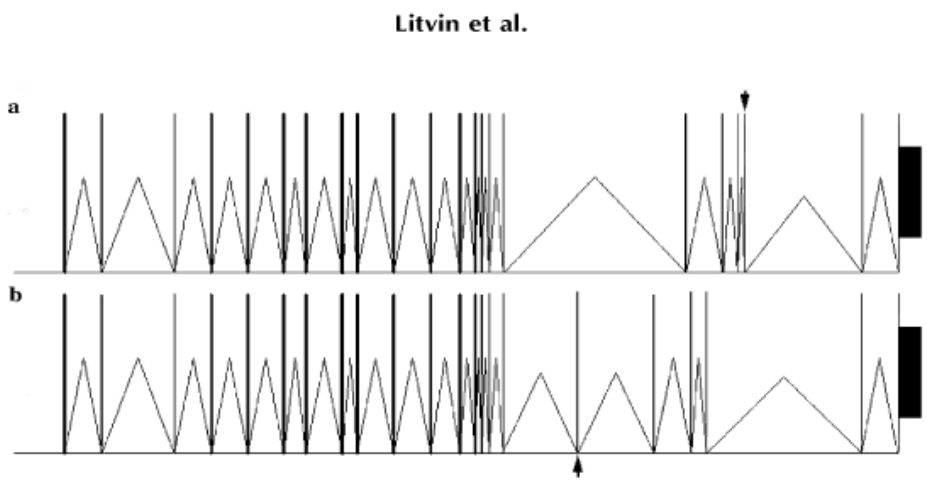



**Figure 7:** Exon-intron arrangement for Periostin **(a)** and PLF **(b)**. The arrow in **(a)** corresponds to exon 21 and the arrow in **(b)** corresponds to exon 17.[20] *Figure from Litvin et al. 2004.*

Recently it was shown that expression of Periostin is regulated by PTH. PTH is a calcium regulation hormone and can stimulate both bone formation and bone resorption. In small intermittent amount it stimulates bone formation, and in that manner it can be used as a anabolic drug to treat diseases like osteoporosis (see Fig. 5). PTH acts by increasing bone remodeling through up-regulation of Periostin in the bone. Majority of the drugs used today to treat osteoporosis focus on inhibiting the resorption by osteoclasts (e.g. bisphosphonates).[5,23,38] Therefore further knowledge about factors like Periostin could be interesting in pharmaceutical perspective.

Recent studies have shown that synergistic use of various BMPs (e.g. BMP-2, BMP-5 and BMP-6) might improve effective bone formation in clinical setting.[6] It has also been shown that TGF-β increases expression of Periostin in osteoblasts.[19] The aim of this research is divided into two parts: 1) To investigate whether there is a collaboration (synergy or potentiating effect) between BMP-2 and Periostin/PLF during osteoblastic differentiation, 2) To investigate whether there is a correlation between expression level of Periostin/PLF and the inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

In this study cells were transfected with pcDNA 3.1 Periostin expression plasmid or small interfering RNA (siRNA) of Periostin/PLF to clarify the role of Periostin/PLF in BMP-2 induced osteoblastic differentiation. These experiments were done in both MC3T3-E1 cells (mouse calvaria-derived osteoblast-like cell line, used as pre-osteoblasts) and C2C12 cells (as pluripotent cells which are able to differentiate in osteoblasts when treated with BMP-2).

# 5. Materials and Methods

## 5.1. Reagents and antibodies

Transfection reagents: Lipofectin® and Lipofectamin™2000 (Invitrogen), JetPRIME (Polyplus transfection Inc., NY, USA), TurboFect™ (Fermentas).

Materials for siRNA obtained from Santa Cruz biotechnology, Inc: Control siRNA-A sc-37007, Periostin siRNA (m) sc-61325, siRNA Dilution Buffer sc-29527.

Antibodies used in Western blot analysis are listed as follows: 1) Rabbit, anti-actin (Sigma-Aldrich), 2) anti-Periostin (H-300): sc-67233 (Santa Cruz biotechnology, inc.), 3) HA-tag (6E2) Mouse mAb #2367 (Cell signaling technology), 4) Anti-rabbit IgG, HRP-linked antibody (Cell signaling technology), 5) Goat anti-mouse IgG HRP (HAF007) (R&D Systems).

Primers for real time reverse transcriptase quantitative polymerase chain reaction (real time RT-qPCR) were purchased from Microsynth, Switzerland.

## 5.2. Cell cultures

### 5.2.1. C2C12 cells

The mouse C2C12 cell line was used as pluripotent cells, a cell line that is able to differentiate into osteoblast when treated with BMP-2. C2C12 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% Fetal Bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin and 2 mM L-Glutamine (obtained from Invitrogen). The cells were incubated at 37°C in 5% $CO_2$ in humidified air. The medium was changed every 2 days.

### 5.2.2. MC3T3-E1 cells

The mouse pre-osteoblast cell line MC3T3-E1 was maintained in proliferation medium consisting of modified Eagle's medium alpha (MEM-α) with 10% FBS. For the experiments the medium was changed to differentiation medium consisting of MEM-α supplemented with 10 % FBS, 50 µg/mL ascorbic acid, 100 U/mL penicillin and 100 U/mL streptomycin. The cells were incubated at 37°C in 5% $CO_2$ in humidified air. The medium was changed every 2 days.

## 5.3. Transient transfection

First, four different transfection reagents were used to see which one was the most effective for the two cell lines that were used. After that Lipofectin® (Invitrogen) was chosen to be used for experiments. The orginal method for transfection with Calcium Phosphate ($CaCl_2$) was also used, following a protocol from Flemington Lab (New Orleans, LA).

Before transfection with siRNA and expression plasmid the medium was changed to antibiotic free medium, DMEM with L-glutamine and 10% FBS for C2C12 cells and alpha-MEM with 10% FBS for MC3T3-E1 cells. Lipofectin® reagent (Invitrogen) was used for transfection according to the manufacturer's recommendations.

#### 5.3.1. Transient transfection with pcDNA 3.1 Periostin/PLF (over-expression)

Transfected C2C12 cells and MC3T3-E1 cells were used to measure the effect of Periostin/PLF on osteoblastic differentiation stimulated by BMP-2 and/or TNF-α. The cells were about 60% confluent at the time of transfection. Transient transfection with expression plasmid was used to over-express Periostin/PLF in C2C12 cells and MC3T3-E1 cells. Different concentrations of pcDNA 3.1 Periostin/PLF plasmid were used (0.2 µg/well on 48 well plates, 0.1-10 µg/well on 24 well plates and 2 µg/well on 12 well plates). After 7-24 hours of culture the medium was changed to normal growth medium with antibiotics (D-MEM for C2C12 and MEM-α for MC3T3-E1). Then the cells were treated with BMP-2 and TNF-α and incubated at 37°C (as described below). The amount of protein was determined with Western Blot. To determine the effect of Periostin/PLF on osteoblastic differentiation ALP, a marker of osteoblastic differentiation, was measured (as described below).

#### 5.3.2. Transient transfection with siRNA of Periostin/PLF (Silencing)

Transfected C2C12 cells and MC3T3-E1 cells were used to measure the effect of Periostin/PLF on osteoblastic differentiation stimulated by BMP-2 and/or TNF-α. Transfection with siRNA Periostin/PLF was used to silence/knock down Periostin/PLF in C2C12 cells and MC3T3-E1 cells. The cells were about 60% confluent at the time of transfection. 10 pmols/well of siRNA Periostin/PLF was used on 48 well plates, 20 pmols/well on 24 well plates and 40 pmols/well on 12 well plates. After 7-24 hours of culture the medium was changed to normal growth medium with antibiotics (D-MEM for C2C12 and MEM-α for MC3T3-E1). The cells were then treated with BMP-2 and TNF-α and incubated at 37°C (as described below). The amount of protein was determined with Western Blot. To determine the effect of Periostin/PLF on osteoblastic differentiation ALP, a marker of osteoblastic differentiation, was measured (as described below).

### 5.4. Treatment with BMP-2 and TNF-α

Both transfected and non-transfected MC3T3-E1 and C2C12 cells were incubated at 37°C with different concentration of BMP-2 and TNF-α (125 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL, 2000 ng/mL of BMP-2 and 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL of TNF-α). After treatment the plates were gently rocked back and forth to achieve even distribution of the complexes. Levels

of Periostin were determined with Western blot analysis (as described below). To determine the effect of BMP-2, TNF-α and BMP-2/TNF-α combined with PLF/Periostin on osteoblastic differentiation ALP, a marker of osteoblastic differentiation, was measured (as described below).

**5.5. Western blot analysis**

Both C2C12 cells and MC3T3-E1 cells which were used for Western blot analysis had been incubated at 37°C for 48 hours after stimulation with BMP and TNF-α. Cells were lysed in Radioimmunoprecipitation assay buffer (RIPA buffer) with protease inhibitor (diluted 1:1000) and phosphatase inhibitor (diluted 1:1000). Proteins were fractionated into a 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred with electroblotting to polyvinylidene difluoride (PVDF) membranes (Millipore, Switzerland). Pre-stained molecular weight markers were used as standards. Blocking of non-specific binding was achieved by placing the membranes in 5% milk in Tris-Buffered Saline Tween (TBST) for 1 hour. After washing once with TBST, the membranes were incubated at room temperature for 1 hour with the primary antibody (rabbit anti-periostin) in bovine serum albumin (BSA) and then stored over night at 4°C. The membranes were rinsed three times with TBST and then exposed to peroxidase-linked secondary antibody (anti-rabbit) in 5% milk in TBST for 1 hour. After rinsing the membranes once with TBST and then again three times for 5 minutes in a clean box, chemiluminescent detection was performed by incubating the membranes with chemiluminescence reagent (Lumi-light Western blotting Substrate 1 and 2 from Roche) and visualization using autoradiography. The process was always repeated for the structural protein actin and for HA (HA is a part of the pcDNA 3.1 Periostin/PLF plasmid) were it applied. Rabbit anti-actin antibody was used to normalize the Periostin/PLF signal versus the amount of protein loading. Membranes that were reprobed had been stripped in stripping buffer (62,5 mM tris HCL (pH 6,8) in 2% SDS with 100 mM β-marcaptoethanol).

The antibodies used are listed as follows: Rabbit anti-Periostin (diluted 1:1000 in 5% BSA); Rabbit anti-actin produced in goats (diluted 1:1000 in 5% BSA), anti-rabbit peroxidase-linked secondary antibody (diluted 1:2500 in 5 % milk); mouse anti-HA (diluted 1:1000 in 5% BSA) and anti-mouse peroxidase-linked secondary antibody (diluted 1:2000 in 5% milk).

**5.6. Alkaline phosphatase measurement**

Both C2C12 cells and MC3T3-E1 cells which were used for ALP measurement had been incubated for at least 72 hours at 37°C after treatment with BMP-2 and/or TNF-α. Each condition of BMP-2

and TNF-α was done in triplicates. The cells were rinsed with Dulbecco's Phosphate Buffered Saline IX (DPBS IX) and then lysed in 250-500 µl of Buffer A (0.56 M 2-amino-2-methyl-propan-1-ol in water with 1N NaOH, pH 10) by keeping the plates for 30 minutes at a rocker at room temperature. The cells were then removed from the bottom of each well with a pipette and added to new eppendorf glasses. Each sample was homogenized with OMNI-MIX (low position) for about 10 sec.

#### 5.6.1. ALP activity

Each sample was mixed with Buffer B (74 mg 20mM Disodium p-nitrophenyl phosphate (371 gr/mol) and 40 µl 4mM $MgCl_2$ in 10 mL of Buffer A) and incubated at 37°C for 15-20 minutes. Then 1 N NaOH was added to each sample. The optical density was measured at 405 nm with a microplate reader (SynergyHT, BioTek).

#### 5.6.2. Protein concentration

Each sample was mixed with Coomassie Blue. BSA standard curve was generated with 4, 6, 8 and 10 µg/mL BSA in duplicates and two blank controls. Optical density was measured at 595 nm with a microplate reader (SynergyHT, BioTek).

### 5.7. Real time RT-qPCR analysis of Periostin, PLF and Runx2

To determine the expression of Periostin, PLF and Runx2 in C2C12 which had been stimulated with BMP-2 or BMP-2/TNF-α, mRNA from C2C12 cells were analyzed by real time RT-qPCR. The C2C12 cells were stimulated with BMP-2 500 µg/mL and/or TNF-α 10 ng/mL and incubated at 37°C for 48 hours. Purification of total RNA from C2C12 cell culture was achieved using RNeasy Mini Kit according to the manufacturer's (Qiagen) recommendation. The RNA quality was checked by measuring RNA concentration using a spectrophotometer (NanoDrop®, ND-1000) and verified by agarose gel electrophoresis. For each sample, 5 µl total RNA was reverse transcribed into cDNA by incubation at 25°C for 10 minutes and then at 42°C for 60 minutes in a volume of 50 µl using First Strand cDNA Synthesis Kit for RT-qPCR as instructed by the manufacturer (Roche). DNA concentration was measured using a spectrophotometer (NanoDrop®, ND-1000) and verified by agarose gel electrophoresis. 1 µl aliquots of the cDNA were amplified in 28 µl PCR reaction mixture containing 2 µl primer set (20 mM) using Quantifast SYBR Green PCR kit from Qiagen.

For amplification the following specific primers were purchased from Microsynth, Switzerland and are listed as follows, forward (F) and reverse (R): Mouse PLF F: 5'-GATAAAATACATCCAAATCAAGTTTG-3'; Mouse PLF R: 5'-CGTGGATCACTTCTGTCACCGTTTCGC-3'; Mouse Periostin F: 5'-

CTGAAAAACAGACTCGGGAAGAACG-3'; Mouse Periostin R: 5'-AAACTCTGTGGTCTGGCCTCTGGG-3'; Mouse Runx2 F: 5'-GAACTACTCCGCCGAGCTC-3'; Mouse Runx2 R: 5'-GGAGGGGTAAGACTGGTCAT-3'. The primers for PLF are located on either side of exon 17, and the primers for Periostin are located on either side of exon 21. Real time RT-qPCR was performed using ICycler® (BioRad) under the following conditions: initial denaturation at 95°C for 5 minutes, amplification through 45 cycles of 95°C for 10 seconds followed by annealing and extension at 60°C for 30 seconds, followed by a final extension step at 60°C for 35 minutes and 30 seconds. Real time RT-qPCR was performed in triplicates, except in duplicates for PLF:control and PLF:BMP-2 due to volume loss when pipetting. The expected product size for Periostin was 304 base pairs (bp), PLF was 225 bp, Runx2 was 319 bp and 425 bp for Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Periostin and PLF gene expression were normalized to the control and GAPDH, which was used as house keeping gene.

### 5.8. Statistical analysis

All analysis were carried out using the statistical software R. Analysis of variance (ANOVA) was used to compare difference in mean ALP activity after transfection with pcDNA 3.1 Periostin plasmid in the four groups (control, 0.1 μg, 1 μg and 10 μg). The null hypothesis that the means of the four groups are equal was rejected (p-value <0.05). Post analysis, to compare levels against each other, was carried out using Tukey test. Unpaired student's T-test was used to determine whether changes in ALP activity after transfection with siRNA Periostin were significant. P-value <0.05 was considered significant for all analysis.

## 6. Results

### 6.1. BMP-2 increases expression of Periostin protein level and ALP activity

The experiments were done in both C2C12 cells and MC3T3-E1 cells. BMP-2 increases Periostin protein expression as determined by Western blot (see Fig. 8). Corresponding increase in ALP activity was recorded (see Fig. 9).

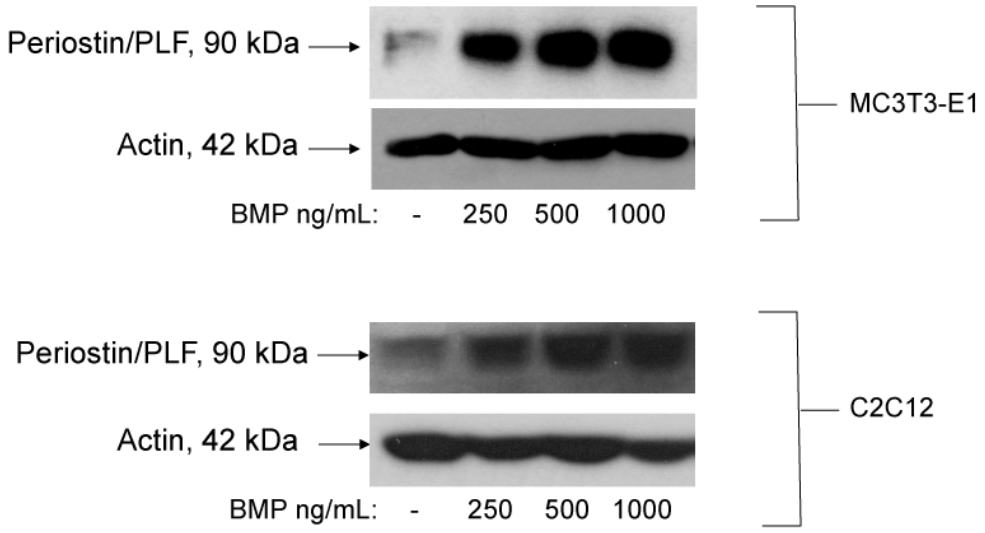



**Figure 8.** BMP-2 increases protein expression of Periostin in MC3T3-E1 cells and C2C12 cells. Amount of protein was determined with Western blot analysis after 48 hours of stimulation with 250 ng/mL, 500 ng/mL, 1000 ng/mL BMP-2 respectively. BMP-2 increases expression of Periostin dose dependently in both cell lines. Although it is more obvious in MC3T3-E1, there is a clear difference between non-treated C2C12 cells and cells treated with 1000 ng/mL BMP-2. The difference in these two cell lines is due to the origin of the cells. Western blot for actin was used as loading control. The experiment was performed in triplicates with the same results.

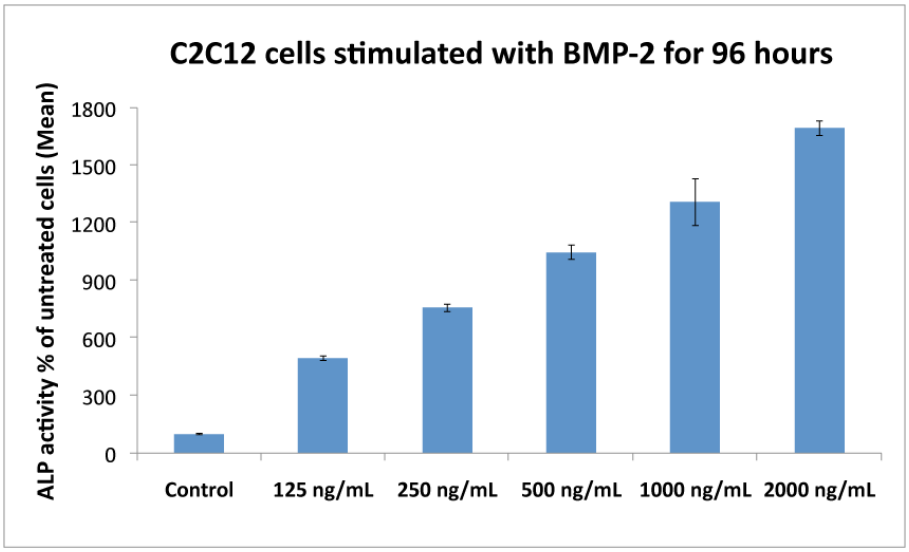



**Figure 9.** BMP-2 increases ALP activity dose dependently. ALP activity was measured after 96 hours of stimulation with BMP-2. Values are represented as mean ± standard deviation. The experiment was performed in triplicates with the same results.

## 6.2. BMP-2 increases expression of Periostin and PLF mRNA in C2C12 cells

Real time RT-qPCR was used to analyze Periostin and PLF mRNA expression in C2C12 cells after 48 hours of stimulation with 500 ng/mL BMP-2. Periostin and PLF mRNA expression was dramatically increased after stimulation with BMP-2 as shown in Figure 10.

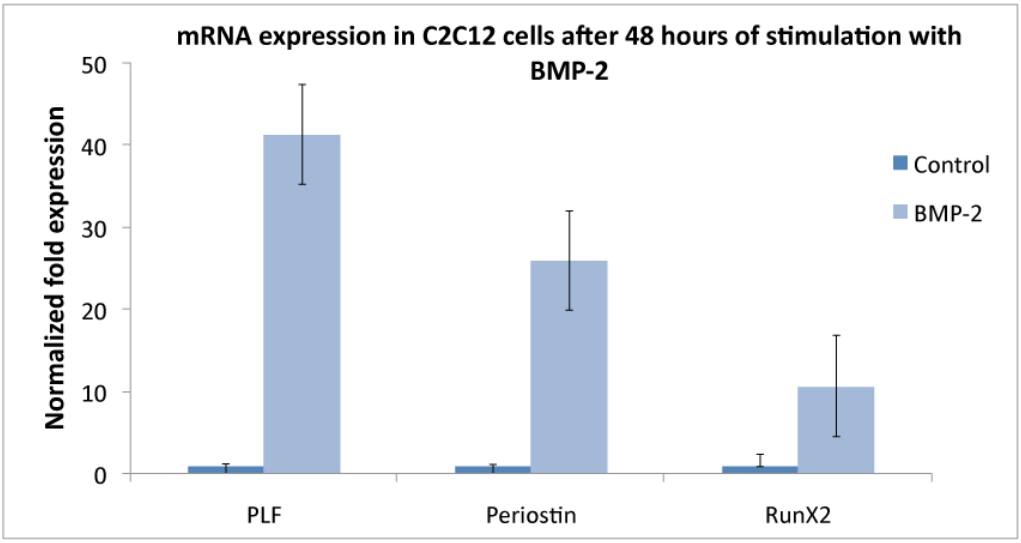



**Figure 10.** RT-qPCR analysis demonstrating Periostin, PLF and Runx2 mRNA expression levels normalized to GAPDH and control in C2C12 cell line. Values are represented as mean ± standard deviation. After 48 hours of stimulation with BMP-2, C2C12 cells expressed Periostin mRNA about 25 times higher than in the control and PLF mRNA levels were about 40 times higher than in the control. mRNA expression of Runx2 (master gene of osteoblastic differentiation) was also higher after stimulation with BMP-2. Measuring runx2 mRNA levels is one way to measure osteoblastic differentiation.

## 6.3. TNF-α decreases expression of Periostin protein level and ALP activity

Periostin protein expression in C2C12 cells and MC3T3-E1 cells is decreased by TNF-α (see Fig. 11). Corresponding decrease in ALP activity was recorded. When cells were treated both with BMP-2 and TNF-α, TNF-α achieved to block the effect of BMP-2 on osteoblastic differentiation (see Fig 12). Even though Periostin/PLF protein expression decreases after treatment with TNF-α, Periostin/PLF mRNA expression is not reduced after treatment with TNF-α (see Fig 13).

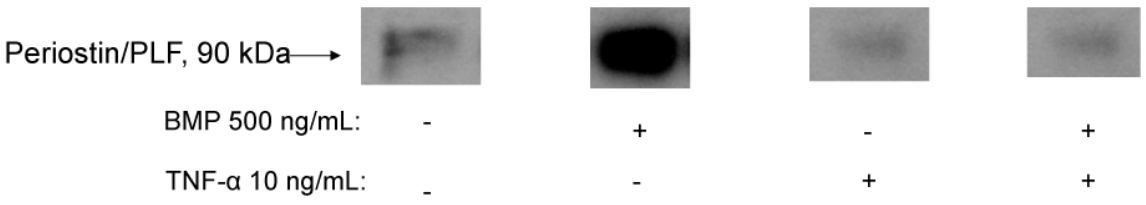



**Figure 11.** TNF-α decreases protein expression of Periostin/PLF. Amount of protein was determined with Western blot analysis after 48 hours of stimulation with 500 ng/mL BMP-2 and/or 10 ng/mL TNF-α. When treated both with BMP-2 and TNF-α a dramatic reduction can be seen in the amount of Periston/PLF protein expressed. Western blot for actin was used as loading control. The experiment was performed in triplicates with the same results and the same trend was seen in C2C12 cells (data not published).

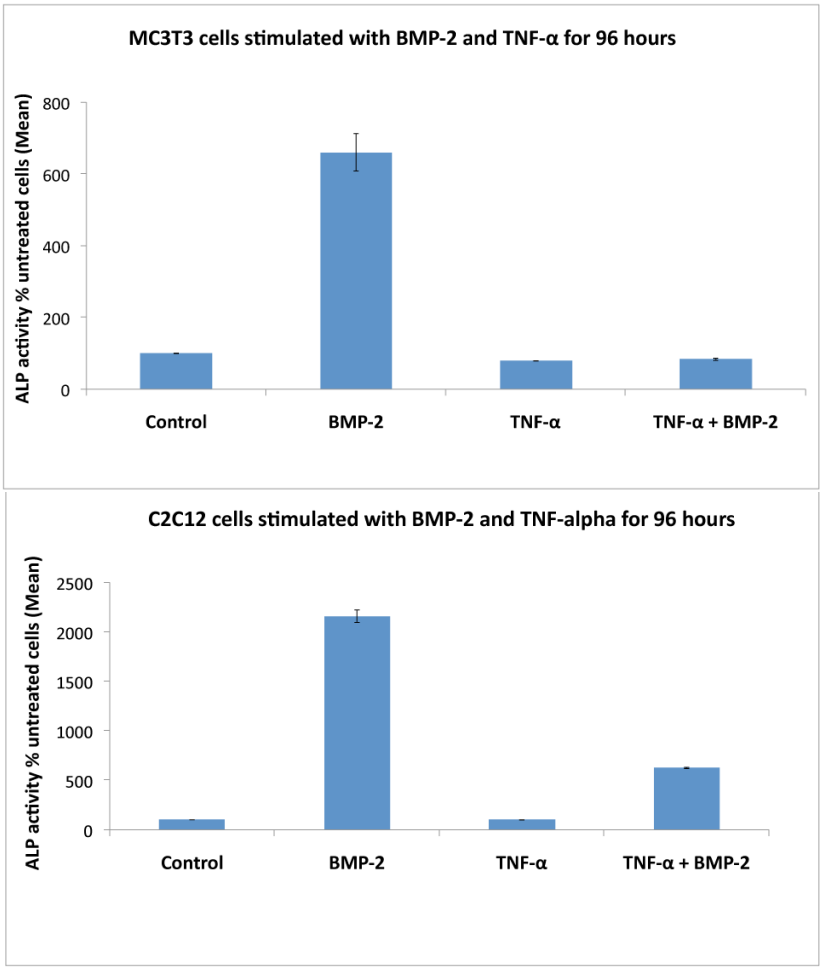



**Figure 12.** TNF-α is a inhibitor of BMP-2 induced osteoblastic differentiation. Exactly the same trend can be seen with C2C12 and MC3T3-E1 cells. ALP activity was measured after 96 hours of stimulation with 500 ng/mL BMP-2 and 10 ng/mL TNF-α. Values are represented as mean ± standard deviation. The experiment was performed in triplicates with the same results.

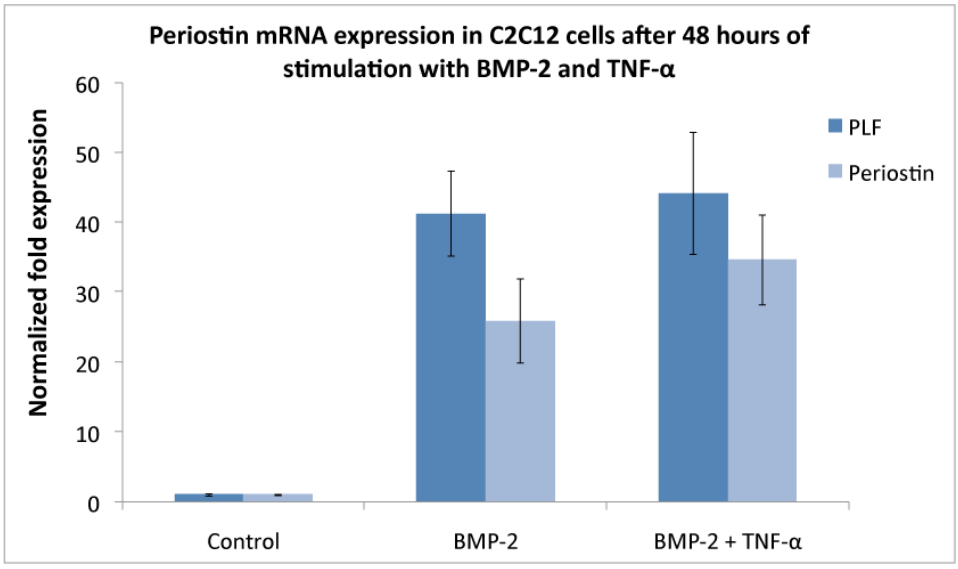



**Figure 13.** RT-qPCR analysis demonstrating Periostin and PLF mRNA expression levels normalized to GAPDH and control in C2C12 cell line. Values are represented as mean ± standard deviation. Similar expression of Periostin and PLF were demonstrated after stimulation with BMP-2 alone and after treatment with both BMP-2 and TNF-α. TNF-α is not inhibiting the effect of BMP-2 on Periostin and PLF mRNA expression. Therefore it is likely that the inhibitory effect of TNF-α is during the translation process. The remaining experiments focused on the protein expression.

## 6.4. The effects of BMP-2 and TNF-α are dose dependent

The increase in ALP activity after treatment with BMP-2 is dose dependent in both C2C12 cells (Fig. 14) and MC3T3-E1 cells (Fig. 15), although the ALP activity in MC3T3-E1 cells soon reaches plateau. The difference in these two cell lines is due to the origin of the cells. The inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation is also dose dependent as shown in Figure 14.

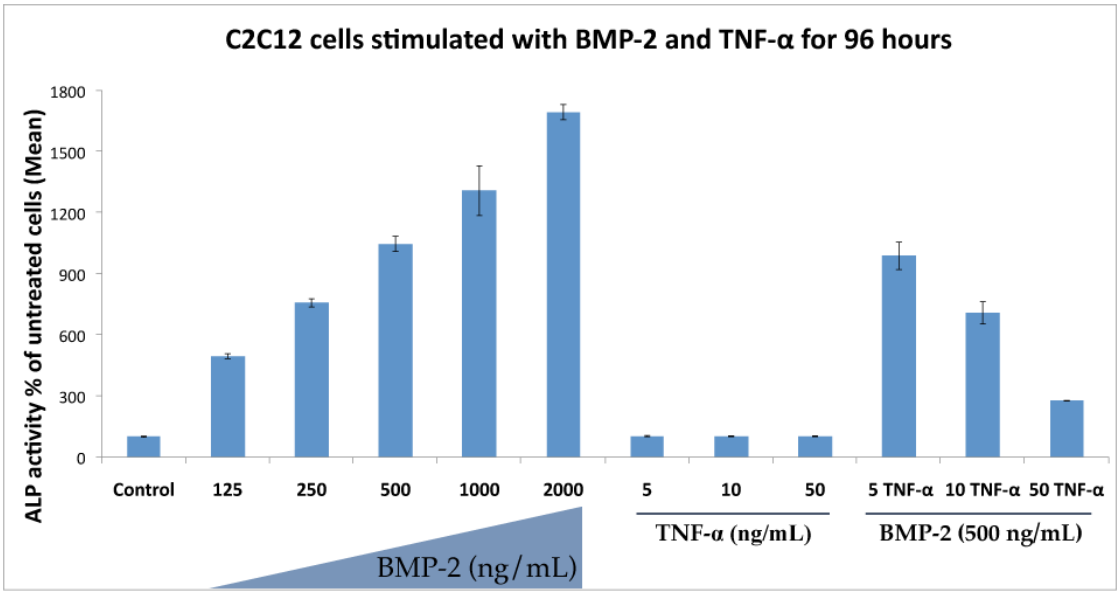



**Figure 14.** The inhibitory effect of TNF-α and stimulation effect of BMP-2 are dose dependent. ALP activity was measured after 96 hours of stimulation with BMP-2 and/or TNF-α. The higher the amount of TNF-α along with the same amount of BMP-2 resulted in lower ALP activity. Values are represented as mean ± standard deviation.

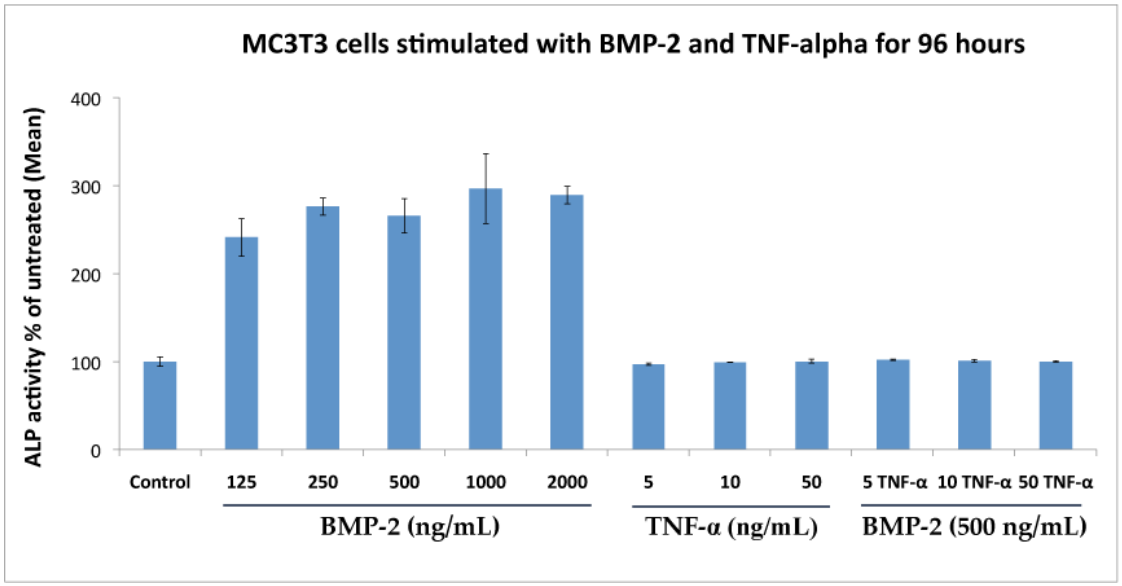



**Figure 15.** The ALP activity in MC3T3-E1 cells reaches plateau after stimulation with relatively low amount of BMP-2. No difference in ALP activity can be seen after treatment with different doses of TNF-α. MC3T3-E1 (pre-osteoblasts) needs lower amount of BMP-2 to become osteoblasts than C2C12 cells (pluripotent cells). ALP activity was measured after 96 hours of stimulation with BMP-2 and/or TNF-α.

## 6.5. Transfection with pcDNA 3.1 Periostin increases Periostin/PLF protein expression

After transient transfection with the expression plasmid pcDNA 3.1 Periostin cells were stimulated with BMP-2 or TNF-α for 48 hours. Expression of Periostin/PLF is increased after transfection with the expression plasmid in both MC3T3-E1 cells (Fig. 16A) and C2C12 (Fig. 16B).

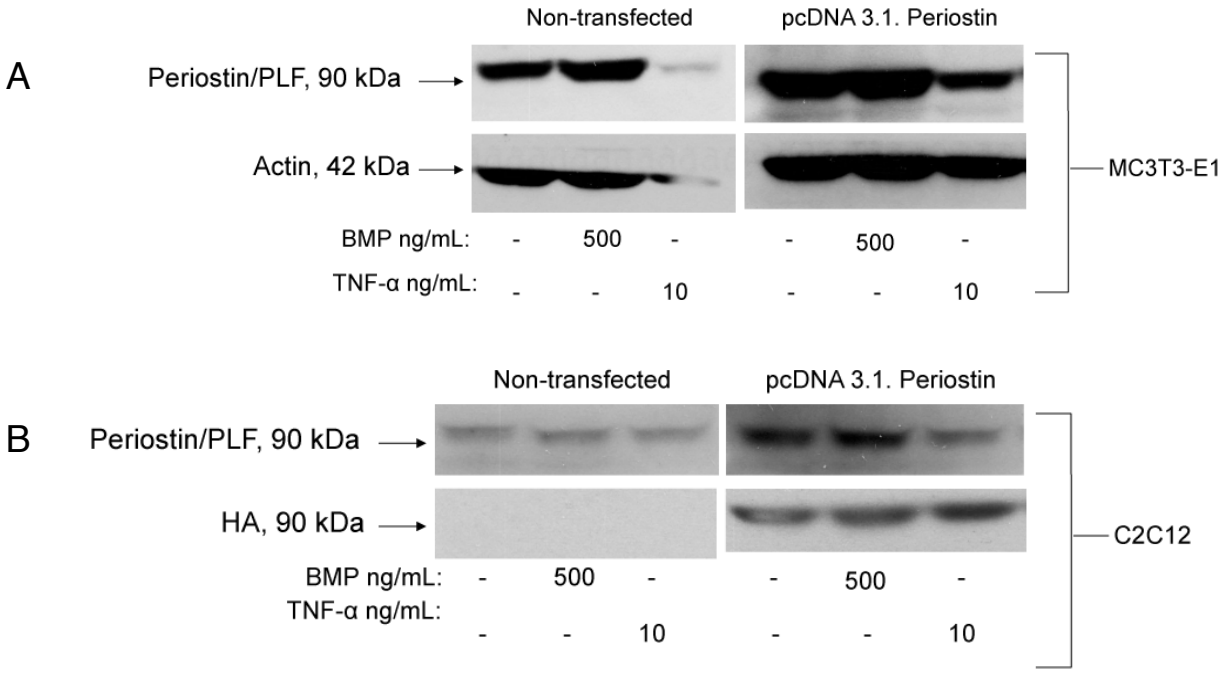



**Figure 16.** Periostin/PLF over-expression in cells transfected with pcDNA 3.1 Periostin resulted in more intense expression of Periostin/PLF compared to non-transfected cells. Amount of protein was determined with Western blot analysis after 48 hours of stimulation with 500 ng/mL BMP-2 or 10 ng/mL TNF-α. The expression was increased both in control and in cells treated with BMP-2 or TNF-α. There is no difference in the profile for non-transfected and transfected cells, BMP-2 is still increasing Periostin/PLF expression and TNF-α is still decreasing Periostin/PLF expression. The difference is just in the amount of protein expressed. Western blot for actin was used as loading control. HA was used as control for cells transfected with pcDNA 3.1 Periostin. HA is a part of the pcDNA 3.1 Periostin plasmid (HA was included in the primers used in the cloning process of the plasmid). When HA is expressed the transfection was successful.

## 6.6. Transfection with pcDNA 3.1 Periostin substantially increases BMP-2 stimulated ALP activity

Transient transfection with pcDNA 3.1 Periostin plasmid (over-expression) substantially increased BMP-2 stimulated ALP activity compared to non-transfected cells (see Table 1 and Figure 17). The effect of the transfection with pcDNA 3.1 Periostin plasmid on ALP activity was dose dependent (see Figure 17).

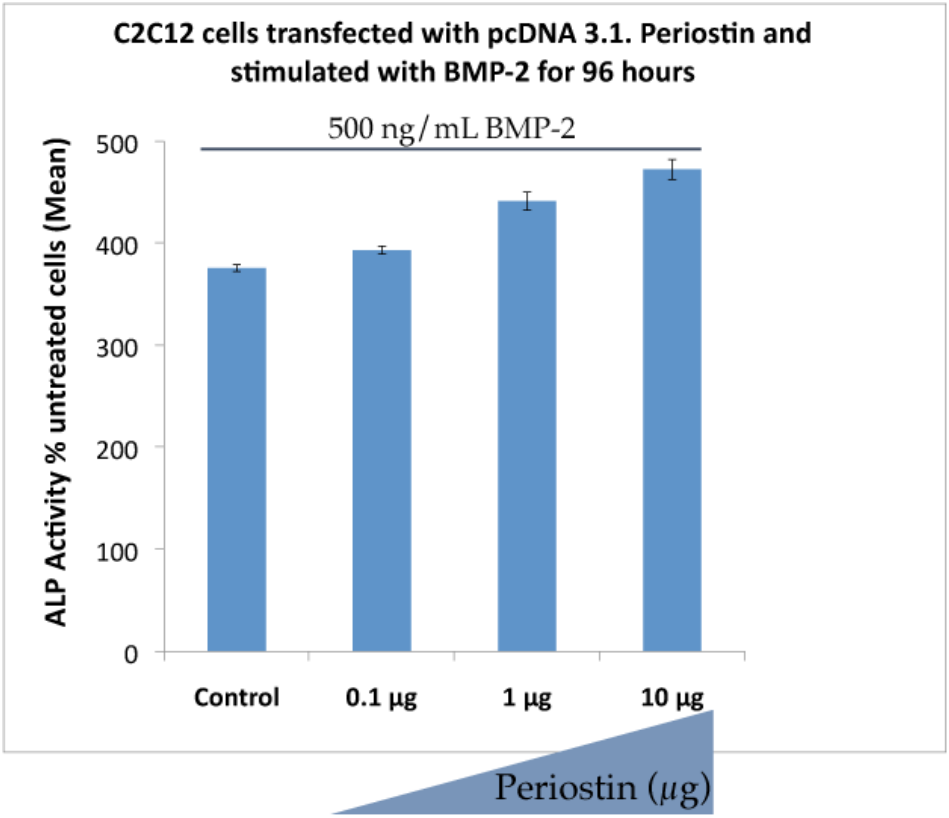



**Figure 17.** BMP-2 stimulated ALP activity was substantially increased by over-expression of Periostin/PLF expression in cells using pcDNA 3.1 Periostin plasmid. ALP activity was measured after 96 hours of stimulation with BMP-2. Control refers to non-transfected cells. The effect of over-expression with pcDNA 3.1 Periostin plasmid is substantially dose dependent. Values are represented as mean ± standard deviation. For p-values, see Table 1.

| | Difference in means | Lower confidence interval | Upper confidence interval | P-value |
|---|---|---|---|---|
| Control vs. 0.1 µg | 17.62 | -1.05 | 36.30 | 0.064 |
| Control vs. 1 µg | 65.73 | 47.06 | 84.41 | < 0.001 |
| Control vs. 10 µg | 96.65 | 77.97 | 115.32 | < 0.001 |
| 0.1 µg vs. 1 µg | 48.11 | 29.44 | 66.78 | < 0.001 |
| 0.1 µg vs. 10 µg | 79.02 | 60.35 | 97.70 | < 0.001 |
| 1 µg vs. 10 µg | 30.91 | 12.24 | 49.59 | 0.003 |

**Table 1.** ANOVA was used to compare any difference in BMP-2 stimulated mean ALP activity after transfection with pcDNA 3.1 Periostin plasmid in the four groups (control, 0.1 µg, 1 µg and 10 µg pcDNA 3.1 Periostin plasmid). Post analysis, to compare levels against each other, was carried out using Tukey test. The changes in ALP activity are significant in all cases, except when 0.1 µg pcDNA 3.1 Periostin is compared to control. Confidence intervals are on the 95% level.

## 6.7. Transfection with siRNA Periostin/PLF reduces Periostin/PLF protein expression

After transient transfection with siRNA Periostin/PLF cells were stimulated with BMP-2 and/or TNF-α for 48 hours. Expression of Periostin/PLF is decreased after transfection with siRNA, especially in control and in cells stimulated with BMP-2 (see Fig. 18).

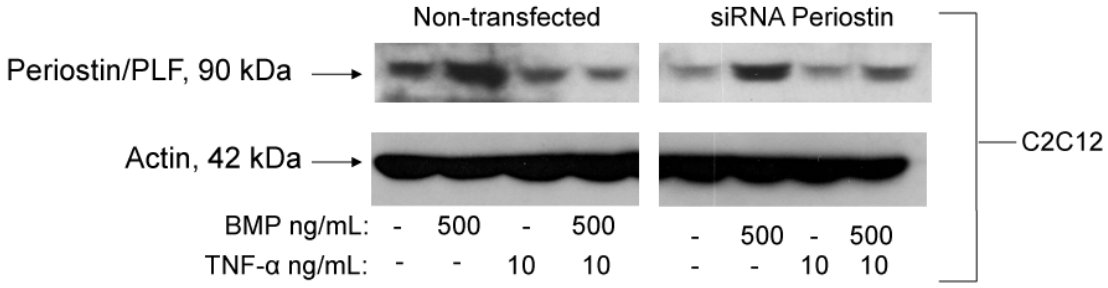



**Figure 18.** Silencing of Periostin/PLF in cells transfected with siRNA Periostin/PLF resulted in reduced expression of Periostin/PLF compared to non-transfected cells. Amount of protein was determined with Western blot analysis after 48 hours of stimulation with 500 ng/mL BMP-2 and/or 10 ng/mL TNF-α. The expression was decreased both in control and in cells treated with BMP-2 or TNF-α. Western blot for actin was used as loading control.

## 6.8. Transfection with siRNA Periostin/PLF substantially decreases BMP-2 stimulated ALP activity

BMP-2 stimulated expression of ALP activity was substantially ($p<0.001$) reduced by silencing of Periostin/PLF expression in cells using siRNA as shown in Figure 19.

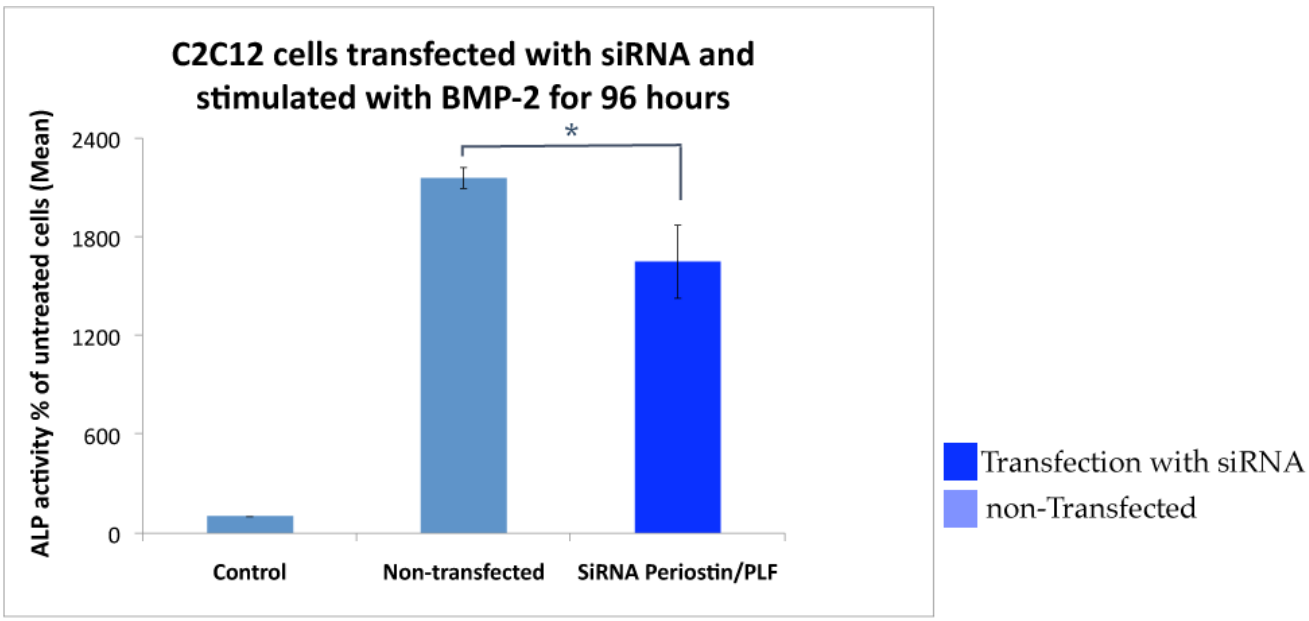



**Figure 19.** BMP-2 stimulated ALP activity was substantially (*$p < 0.001$) decreased with silencing of Periostin/PLF expression in cells using siRNA Periostin/PLF compared to non-transfected cells. ALP activity was measured after 96 hours of stimulation with BMP-2. Control refers to non-stimulated, non-transfected cells. Values are represented as mean ± standard deviation.

## 6.9. TNF-α still inhibits BMP-2 induced osteoblastic differentiation after both over-expression and silencing of Periostin/PLF

Over-expression of Periostin/PLF with expression plasmid and silencing of Periostin/PLF with siRNA did not result in restored ALP activity in cells treated with BMP-2 and TNF-α as shown in Figure 20 and 21 respectively. Silencing and over-expression of Periostin/PLF is just modifying the response to BMP-2 alone, not when BMP-2 is compared with TNF-α.

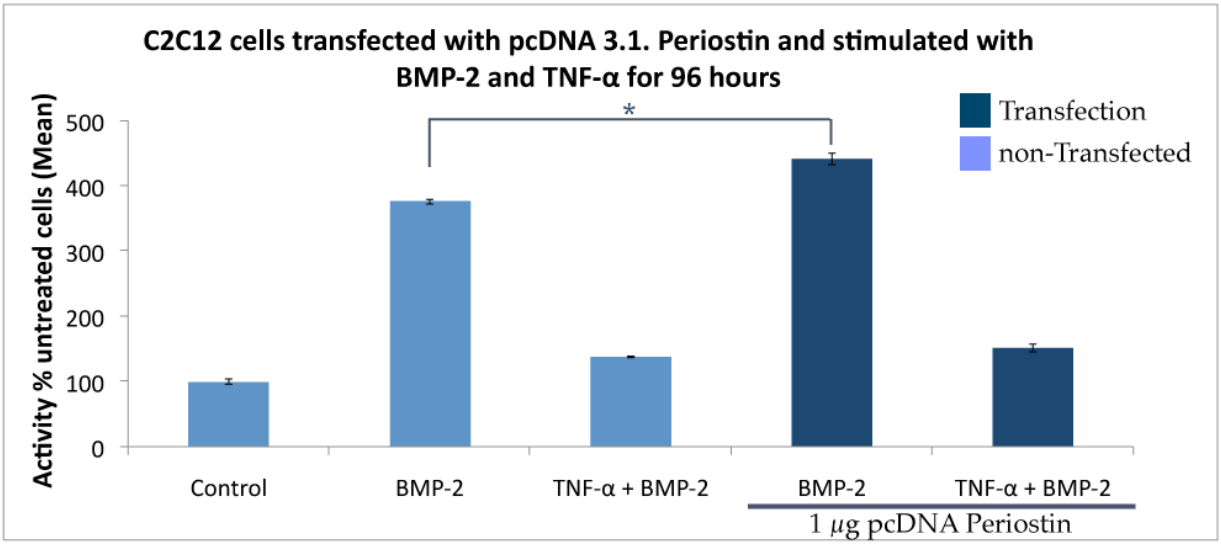



**Figure 20.** TNF-α inhibits BMP-2 stimulated osteoblastic differentiation even after over-expression of Periostin/PLF with pcDNA 3.1 Periostin plasmid. Over-expression with Periostin is only modifying the response to BMP-2 alone (*p < 0.001). ALP activity was measured after 96 hours of stimulation with 500 ng/mL BMP-2 and/or 10 ng/mL TNF-α. Values are represented as mean ± standard deviation.

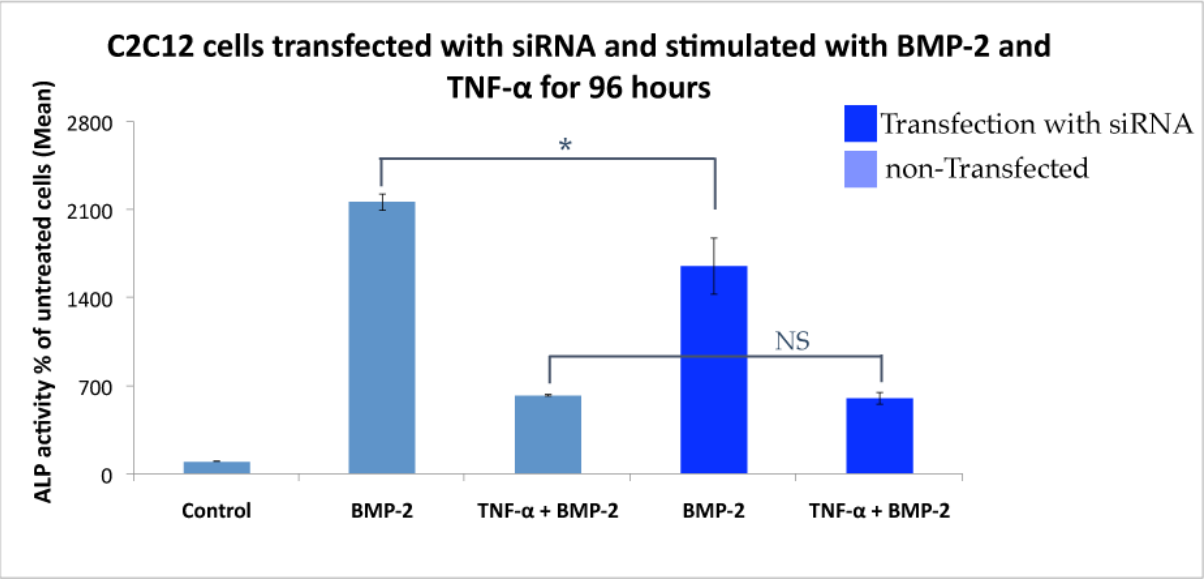



**Figure 21.** TNF-α still inhibits BMP-2 stimulated osteoblastic differentiation after silencing of Periostin/PLF using siRNA Periostin/PLF. Over-expression with Periostin is only modifying the response to BMP-2 alone (*p < 0.001, NS=Non significant). ALP activity was measured after 96 hours of stimulation with 500 ng/mL BMP-2 and/or 10 ng/mL TNF-α. Values are represented as mean ± standard deviation.

# 7. Discussion

## 7.1. Objective of the study

The main objective of this study was to investigate Periostin's role in BMP-2 induced osteoblastic differentiation. BMP-2 is known to be one of the most potent growth factors that stimulates osteoblastic differentiation and it was recently shown that Periostin/PLF promotes osteoblastic proliferation and differentiation in vitro.[9,10] Therefore we wanted to know if there is a collaboration between BMP-2 and Periostin/PLF during osteoblastic differentiation. It is also known that TNF-α has a inhibitory effect on BMP-2 induced osteoblastic differentiation.[17] We wanted to know whether Periostin/PLF is involved in this inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

## 7.2. Results compared to previous knowledge

In this study the collaboration between BMP-2 (a member of the TGF-β superfamily) and Periostin/PLF was investigated by determining Periostin/PLF protein expression, Periostin/PLF mRNA expression and BMP-2 stimulated ALP activiy. We have shown that BMP-2 increases expression of ALP activity and Periostin/PLF protein level dose dependently. With higher concentration of BMP-2 in cell culture, more amount of Periostin protein is expressed and higher ALP activity is recorded. Corresponding increase in Periostin/PLF mRNA expression was recorded after stimulation with BMP-2. Our results corresponds to previous published data from experiments in primary osteoblasts, were Periostin expression was increased dose dependently after stimulation with TGF-β.[19] Levels of ALP activity confirms that BMP-2 stimulates osteoblastic differentiation as already published.[9,10]

Over-expression of Periostin with pcDNA 3.1 Periostin plasmid resulted in more amount of Periostin protein expressed and significantly higher BMP-2 stimulated ALP activity. Furhermore, silencing of Periostin/PLF with siRNA Periostin/PLF resulted in lower amount of protein expressed and significantly lower BMP-2 stimulated ALP activity. Based on these results we conclude that Periostin/PLF potentiates the effect of BMP-2 in osteoblastic differentiation. rhBMP-2 is already used in clinical setting. rhBMP-2 is combined with a scaffold that permit its retention and release at the wound site.[12-14] Based on our results, it would be interesting to develop a scaffold that contains combination of Periostin and BMP-2 which is released gradually to induce bone formation, and by that promote more bone formation than with BMP-2 alone. Of course the role of Periostin needs to be studied in more details before this would be possible.

Our results from Western blot analysis and ALP measurements in C2C12 and MC3T3-E1 cell lines confirms that BMP-2 induced osteoblastic differentiation is dose dependently inhibited by TNF-α, as previously published in C2C12 cells.[17]

The correlation between Periostin/PLF and TNF-α has not been studied in details. The role of TNF-α and Periostin/PLF has only been studied in rat model and primary cells in a research that focused on inflammation.[16] In this study we report that TNF-α decreases amount of Periostin/PLF protein expressed in both C2C12 and MC3T3-E1 cells as determined with Western blot analysis. TNF-α also did inhibit the effect of BMP-2 on Periostin/PLF protein expression in both cell lines. Surprisingly, Periostin/PLF mRNA level were similar in C2C12 cells treated with BMP-2 alone and in cells treated both with BMP-2 and TNF-α. This data suggests that the inhibitory effect of TNF-α is during the translation process.

Neither did transfection with pcDNA 3.1 Periostin nor transfection with siRNA periostin restore ALP activity in cells treated with BMP-2 and TNF-α. Over-expression and silencing of Periostin/PLF only affected ALP activity in cells which were just stimulated with BMP-2. Periostin/PLF is therefore not involved in the inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

## 7.3. Advantages of the study

In the beginning of the process different transfection reagents were tested to see which one was the best for the cell lines used (data not published). In all the transfection experiments, the control cells were also treated with the transfection reagent alone (without pcDNA 3.1 Periostin plasmid and siRNA Periostin). This was performed to be sure that the transfection reagent itself was not having any effect on the cells.

## 7.4. Defects of the study

### 7.4.1. Periostin versus PLF

Periostin and PLF differ in the C-terminal region. Periostin contains a specific sequence of 28 aa, between aa 785-812, which is absent in PLF. Similarly, PLF contains a specific sequence of 27 aa, between aa 673-699, which is not found in Periostin. For the Western blot analysis we used anti-Periostin polyclonal antibody (no monoclonal antibody for Periostin/PLF is available) against aa 537-836. Despite this small difference in the aa sequence of Periostin and PLF the antibody should recognize both isoforms. The siRNA used was a pool of three different siRNAs for Periostin and

should also recognize both isoforms. Finally, the plasmid used was PLF not Periostin. This means that we can not be absolutely sure if we have both isoforms or only one expressed.

#### 7.4.2. Western blot: Actin and HA

Actin was used as a loading control in Western blot analysis. In some of the experiments there was a problem with the amount of actin expressed. The loading control was used to be sure that even amount of samples were loaded to the slots in the gel. When there is not the same amount of actin in every sample (like in some of the experiments), one can not be sure that the effect of e.g. BMP-2 or Periostin is really true. Less or more amount of protein expressed can be due to the amount of sample loaded to the slots. This problem we had with actin in some of the experiments was most likely do to the author´s lack of experience in pipetting.

HA was used as a control for cells transfected with pcDNA 3.1 Periostin. HA is a part of the pcDNA 3.1 Periostin plasmid (HA was included in the primers used in the cloning process of the plasmid). When HA is expressed, one can be sure that transfection with pcDNA 3.1 Periostin was successful. Only in one experiment we were able to see the expression of HA by Western blot analysis, although transfection with pcDNA 3.1 Periostin was performed in triplicates. Our hypothesis is that there was something wrong with the anti-HA antibody used.

#### 7.4.3. Not all the experiments were done in triplicates

Due to the short time range of this study, we had to choose which experiments to perform. Therefore not all experiments were done in triplicates. For example, PCR was only performed once and only with C2C12 cells. Furthermore, Western blot and ALP measurement for cells transfected with siRNA were only performed once.

### 7.5. Next step - future experiments

#### 7.5.1. Repeat experiments

As said earlier, some of the experiments were not performed in triplicates. To be sure that the results are accurate, it is necessary to repeat these experiments, especially the Western blot analysis for transfected cells. It would also be necessary to repeat some of the Western blots in which there was a problem with the loading control (actin) and the transfection control (HA) with a new antibody from a different company in the latter case.

#### 7.5.2. Protein purification

Recently Takayama et al managed to produce and purify Periostin protein produced in *Escherichia coli (E. coli)*. Their findings will facilitate future research on the function of Periostin.[39] During the last weeks of this project we started to purify recombinant Periostin protein, produced by *E. coli.*

pET-23a(+) Periostin plasmid (previously cloned) was introduced into *E. coli* competent cells. The competent cells were induced to produce the protein cloned using isopropy-1-thio-β-D-galactopyranoside (IPTG). Selection of clones was achieved using growth medium with Ampicillin, because only the cells with the plasmid inside were resistance to Ampicillin, and were able to grow on the agar plate. The recombinant protein was purified using affinity chromatography. Due to the short time range of this project, this process was not finished (data not published). The next step for this study would be to continue with the protein purification.

The aim of the protein purification is to be able to stimulate cell culture with recombinant Periostin protein, in the presence or not of BMP-2. By stimulation with recombinant Periostin protein you can control more precisely the amount of Periostin protein used, than with the over-expression plasmid. Although cells are transfected with e.g. 10 µg of pcDNA 3.1 Periostin you can not be sure that exactly 10 µg of Periostin really got into the cells.

#### 7.5.3. Distinguish between Periostin and PLF

Some researchers hypothesize that these two isoforms of Periostin may have significant functional consequences, but it has not been examined in details.[20] Therefore, in further experiments it is necessary to distinguish between Periostin and PLF. It would be optimal to obtain a monoclonal antibody specific for Periostin and another specific for PLF. In that manner one can be sure what isoform of Periostin is expressed, and it would give an opportunity to study possible different functions of these two isoforms. For the same reasons, it would be optimal to use siRNA and expression plasmid specific for Periostin and PLF.

#### 7.5.4. Experiments in primary cells

Eventually, all these experiments would have to be done in primary cells. Primary cells have undergone fewer population doublings than continuous cell lines (as were used in this study) and might therefore represent better the functional elements of the bone tissue.

## 7.6. Conclusion

First of all, we have shown that BMP-2 increases Periostin/PLF protein expression and secondly that Periostin/PLF potentiates the effect of BMP-2 in osteoblastic differentiation. We have also shown that TNF-α decreases Periostin/PLF protein expression and confirmed that TNF-α inhibits the effect of BMP-2 on osteoblastic differentiation. Although treatment with TNF-α resulted in lower amount of Periostin/PLF protein expressed, neither did transfection with pcDNA 3.1 Periostin nor transfection with siRNA periostin restore ALP activity in cells treated with BMP-2 and TNF-α.

Therefore we conclude that Periostin/PLF is not involved in the inhibitory effect of TNF-α on BMP-2 induced osteoblastic differentiation.

## 8. Acknowledgments

First of all I'm grateful to my supervisor, Dr. Chafik Ghayor for excellent teaching, advice and help. I really appreciate the opportunity that Dr. Franz E. Weber gave me by welcoming me to the laboratory and the grate facilities I had during the project. I want to thank all the members of Franz's group at the laboratory for cranio-maxillofacial surgery at the University hospital of Zürich for their patient and helpful comments during the project. Especially I want to thank Yvonne Bloemhard for assistance with protein purification and Lindsay Sulzer for assistance with real time RT-qPCR. Finally I want to thank Garðar Sveinbjörnsson for advice with statistical analysis.

## 9. References

1. Karsenty G. Minireview: transcriptional control of osteoblast differentiation. Endocrinology 2001;142:2731-3.
2. Owen TA, Aronow M, Shalhoub V, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 1990;143:420-30.
3. John P. Bilezikian, Raisz LD, Martin TJ. Principles of Bone Biology. Third edition ed: Elsevier; 2008.
4. Bronner F, Farach-Carson MC. Bone formation: Springer; 2004.
5. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville, MD.: U.S. Department of Health and Human Services . Public Health Service. Office of the Surgeon General 2004.
6. Wutzl A, Rauner M, Seemann R, et al. Bone morphogenetic proteins 2, 5, and 6 in combination stimulate osteoblasts but not osteoclasts in vitro. J Orthop Res 2010;28:1431-9.

7. Drake MT, Cremers SC. Bisphosphonate therapeutics in bone disease: the hard and soft data on osteoclast inhibition. Mol Interv 2010;10:141-52.
8. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 2004;22:233-41.
9. Chaudhari A, Ron E, Rethman MP. Recombinant human bone morphogenetic protein-2 stimulates differentiation in primary cultures of fetal rat calvarial osteoblasts. Mol Cell Biochem 1997;167:31-9.
10. Yamaguchi A, Katagiri T, Ikeda T, et al. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 1991;113:681-7.
11. Bessa PC, Casal M, Reis RL. Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). Journal of Tissue Engineering and Regenerative Medicine 2008;2:1-13.
12. Pallua N, Suschek CV. Tissue engineering: From lab to clinic: Springer Heidelberg; 2011.
13. Barr T, McNamara AJA, S·ndor GKB, Clokie CML, Peel SAF. Comparison of the osteoinductivity of bioimplants containing recombinant human bone morphogenetic proteins 2 (Infuse) and 7 (OP-1). Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 2010;109:531-40.
14. McKay WF, Peckham SM, Badura JM. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). International orthopaedics 2007;31:729-34.
15. Nanes MS. Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology. Gene 2003;321:1-15.
16. Rani S, Barbe MF, Barr AE, Litivn J. Role of TNF alpha and PLF in bone remodeling in a rat model of repetitive reaching and grasping. J Cell Physiol 2010;225:152-67.
17. Mukai T, Otsuka F, Otani H, et al. TNF-alpha inhibits BMP-induced osteoblast differentiation through activating SAPK/JNK signaling. Biochem Biophys Res Commun 2007;356:1004-10.
18. Takeshita S, Kikuno R, Tezuka K, Amann E. Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. Biochem J 1993;294 ( Pt 1):271-8.
19. Horiuchi K, Amizuka N, Takeshita S, et al. Identification and Characterization of a Novel Protein, Periostin, with Restricted Expression to Periosteum and Periodontal Ligament and

Increased Expression by Transforming Growth Factor β. Journal of Bone and Mineral Research 1999;14:1239-49.

20. Litvin J, Selim AH, Montgomery MO, et al. Expression and function of periostin-isoforms in bone. J Cell Biochem 2004;92:1044-61.
21. Coutu DL, Wu JH, Monette A, Rivard G-E, Blostein MD, Galipeau J. Periostin, a member of a novel family of vitamin K-dependent proteins, is expressed by mesenchymal stromal cells. Journal of Biological Chemistry 2008;283:17991-8001.
22. Norris RA, Damon B, Mironov V, et al. Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. J Cell Biochem 2007;101:695-711.
23. Fortunati D, Reppe S, Fjeldheim AK, Nielsen M, Gautvik VT, Gautvik KM. Periostin is a collagen associated bone matrix protein regulated by parathyroid hormone. Matrix Biol 2010;29:594-601.
24. Kuhn B, del Monte F, Hajjar RJ, et al. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat Med 2007;13:962-9.
25. Lie-Venema H, Eralp I, Markwald RR, et al. Periostin expression by epicardium-derived cells is involved in the development of the atrioventricular valves and fibrous heart skeleton. Differentiation 2008;76:809-19.
26. Nakazawa T, Nakajima A, Seki N, et al. Gene expression of periostin in the early stage of fracture healing detected by cDNA microarray analysis. J Orthop Res 2004;22:520-5.
27. Litvin J, Blagg A, Mu A, et al. Periostin and periostin-like factor in the human heart: possible therapeutic targets. Cardiovasc Pathol 2006;15:24-32.
28. Ben QW, Jin XL, Liu J, Cai X, Yuan F, Yuan YZ. Periostin, a matrix specific protein, is associated with proliferation and invasion of pancreatic cancer. Oncol Rep 2011;25:709-16.
29. Li JS, Sun GW, Wei XY, Tang WH. Expression of periostin and its clinicopathological relevance in gastric cancer. World J Gastroenterol 2007;13:5261-6.
30. Puppin C, Fabbro D, Dima M, et al. High periostin expression correlates with aggressiveness in papillary thyroid carcinomas. J Endocrinol 2008;197:401-8.
31. Takanami I, Abiko T, Koizumi S. Expression of periostin in patients with non-small cell lung cancer: correlation with angiogenesis and lymphangiogenesis. Int J Biol Markers 2008;23:182-6.
32. Utispan K, Thuwajit P, Abiko Y, et al. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. Mol Cancer 2010;9:13.

33. Tischler V, Fritzsche FR, Wild PJ, et al. Periostin is up-regulated in high grade and high stage prostate cancer. BMC Cancer 2010;10:273.
34. Contie S, Voorzanger-Rousselot N, Litvin J, Clezardin P, Garnero P. Increased expression and serum levels of the stromal cell-secreted protein periostin in breast cancer bone metastases. Int J Cancer 2011;128:352-60.
35. Zhu M, Fejzo MS, Anderson L, et al. Periostin promotes ovarian cancer angiogenesis and metastasis. Gynecol Oncol 2010;119:337-44.
36. Ruan K, Bao S, Ouyang G. The multifaceted role of periostin in tumorigenesis. Cell Mol Life Sci 2009;66:2219-30.
37. Zhu S, Barbe MF, Liu C, et al. Periostin-like-factor in osteogenesis. Journal of Cellular Physiology 2009;218:584-92.
38. Tian Y, Xu Y, Fu Q, He M. Parathyroid hormone regulates osteoblast differentiation in a Wnt/beta-catenin-dependent manner. Mol Cell Biochem 2011.
39. Takayama I, Kii I, Kudo A. Expression, purification and characterization of soluble recombinant periostin protein produced by Escherichia coli. J Biochem 2009;146:713-23.