

Nicotinamide inhibits Heterotopic ossification decreasing Smad 1/5/8 phosphorylation

Ivan Quevedo (✉ equevedo@udec.cl)

Universidad de Concepcion

Fernando Rivas-Valdes

Universidad de Concepcion

Soraya Gutierrez

Universidad de Concepcion

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Abstract

Heterotopic ossification (HO) is a condition in which extra-skeletal bone formation occurs in soft tissues of the body. HO is formed in numerous clinical situations such as burns, neurological damage, post-combat trauma, hip surgery, etc. The most severe condition of heterotopic ossification is the pathology called Fibrodysplasia Ossificans Progressiva (FOP), which is produced by a point mutation in the ACVR1 receptor gene, triggering its constitutive activation and a hyper-activation of the SMAD pathway. Different pharmacological treatments have been used to inhibit the progression of ossification outbreaks, but a low effectiveness has been reported. An alternative therapy is the use of nicotinamide where a delay in the appearance of ossification flare ups has been observed. However, its effect on the SMAD-mediated signaling pathway is not known. In this work we use nicotinamide in culture of C2C12 cells in the presence or absence of the osteoinductive BMP-2 to investigate its effect on osteoblastogenesis. Through western blot, Immunocytochemistry and Real time PCR we investigated the effect of nicotinamide on the SMAD pathway and gene expression of genes involved in the pathology. Our results show that in the presence of nicotinamide a decrease in the osteoblast differentiation and in SMAD complex phosphorylation levels while expression of the ACVR1 BMP-2 receptor and Runx2 are not affected by the treatment. New evidence is provided regarding the mechanism by which nicotinamide acts. These findings could be helpful in the clinic for the treatment of diseases related to HO bone formation.

Introduction

Heterotopic ossification is a debilitating condition where the formation of ectopic trabecular bone, i.e. extra skeletal, occurs in the body's soft tissues [1]. It is presented as a complication of numerous clinical situations such as trauma, traumatic surgeries, in the newborn after vaginal deliveries, neurological injuries (head trauma), severe burns, hip arthroplasty [2], and spinal cord injuries among others [3]. Additionally, this pathological condition is a great concern since it is a serious medical issue in patients that develop ectopic bone as result of post-traumatic combats and represents a challenge for prophylaxis and rehabilitation [4].

This condition causes a decrease in mobility of joints, as well as pain at the site of ossification that is usually accompanied by inflammation of tissue. The disability that occurs because of ossification is variable, but it is estimated that more than 20% of patients will develop a disability such as joint stiffness or chronic pain [1].

Heterotopic ossification can be observed in more than 50% of patients with severe cranial brain injuries 44% of hip surgeries, 14% of arthroscopies, and up to 3% of the adult population with ossification of the posterior spinal ligament and between 0.2% and 4% of births [5].

There are several therapies to prevent heterotopic ossification, including the use of non-steroidal anti-inflammatory drugs that have been relatively successful, such as Etidronate and Indomethacin used in

patients with spinal cord injury, achieving a decrease in recurrence of ossification [6, 7]. However, the impact they have on fracture healing must be considered; It has been reported that prevention of heterotopic ossification using Indomethacin increases the risk of unconsolidated fractures [8]. On the other hand, the use of bisphosphonates as agents that induce the apoptosis of osteoclasts have been questioned by its low effectiveness, in addition to its severe side effects, in such as the generation of mandibular osteonecrosis, which results in other complications [9].

The most severe condition of heterotopic ossification is a rare autosomal mutation Fibrodysplasia Ossificans Progressiva (FOP; MIM 135100) in which excess bone tissue spontaneously forms in ectopic sites forming a second skeleton. The genetic alteration causing FOP occurs in the *ACVR1* gene- R206H- which codes for the BMP-2 growth factor receptor [10]. This mutation produces a constitutive activation of the ACVR1 receptor, which phosphorylates the SMAD 1/5/8 protein complex. The constitutive hyperactivation of ACVR1 induces the osteogenic pathway, triggering the formation of heterotopic bone in extraskeletal sites [11]. Researchers have reported the use of nicotinamide in the treatment of this pathology and observed a delay in the appearance of outbreaks of ossification [12]. Previous reports showed that nicotinamide can prevent the osteoblastogenesis in an *in vitro* model, however, the mechanism by which nicotinamide acts at the level of the canonical pathway of osteogenic signaling Smad is still unknown.

The aim of the present study is to determine the effect of nicotinamide on the differentiation of osteoblasts, particularly on the canonical osteogenic pathway because it has been described that it is altered in FOP. In this study, we report the effect of nicotinamide on the BMP signaling pathway, which is central in the process of bone formation affected in FOP. The evidence presented in this report suggests that the activation of the smad-dependent bone pathway is inhibited by decreasing the phosphorylation levels of the Smad 1/5/8 complex that consequently results in an inhibition of osteoblastogenesis.

Materials And Methods

Cell Culture

The mouse pre-myoblast cell line C2C12, was obtained from European Cell Culture ECCAC. C2C12 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with High glucose (Thermo) containing 10% of fetal bovine serum (Gibco) and antibiotics (100U/mL Penicillin and 100µg/mL of Streptomycin) and L-Glutamine (Thermo) at 37°C

in a humidified atmosphere of 5% CO₂. Cells ($3,5 \times 10^5$) were plated into 100-mm culture

dishes with regular growth medium previously described. At 24 h after plating, regular growth medium was replaced by 0,25% albumin as described [13] and maintained in culture medium depleted of serum for another 24 h.

RNA extraction, and quantitative real-time PCR analysis.

Total RNA was isolated from C2C12 cells in the presence or absence of BMP-2 (Thermo Fisher, PHC7141) and in presence or absence of nicotinamide (Sigma-Aldrich, N0636). Cells were washed with PBS and lysed with TRIzol reagent (Invitrogen), and using the DirectZol RNA mini prep kit (Zymo research, #R2050). After RNA purification, the integrity was assessed by agarose electrophoresis and the quantification was estimated with the Qubit 2.0 instrument (Life Technologies). 2µg of total RNA was used for reverse transcription using M-MLV (Promega) and the product was then used for real-time PCR using the Maxima® SYBR Green/Rox qPCR Master mix 2X (Fermentas Life Sciences Cat. #K0221). PCRs were performed in triplicate for each cDNA the results were averaged. The relative mRNA levels were normalized to the reference Mouse Snrpd3 gene. The reaction was carried out in a Stratagene Mx3000 (Agilent Technologies) thermocycler. PCR primers were designed with Primer-BLAST program [14] using different exon sequences of the corresponding genes (Table 1).

Western Blot analyses

C2C12 cells were exposed to BMP-2 (500 ng/mL) and/or nicotinamide (20 mM) for 0, 0.5, 2 and 4 hours. Then total proteins lysate were extracted for later analyses. Halt Protease and Phosphatase inhibitor cocktail (Thermo Scientific #78440) was used to prevent degradation and dephosphorylation of proteins. Whole cell lysates were separated by SDS-PAGE electrophoresis using NuPAGE 4-12% Bis-Tris precast gel (Invitrogen) and the Bolt Mini Gel Tank System (Life Technologies) according to the instructions of the manufacturer. For immunodetection pSmad 1/5/8 rabbit monoclonal antibody (Cell signaling, Danvers, MA, USA cat# 13820), GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology sc-32233) were used. Anti-rabbit and Anti-mouse IgG conjugated with HRP (Sigma-Aldrich) was used as the secondary antibody respectively. Immunoreactivities were detected by Pierce ECL Western Blotting Substrate (Thermo scientific).

Cell culture staining

Cells were seeded into a 24-well plate and cultured for 24 and 48 hrs in the same conditions described previously. Cultures were fixed with 4% Paraformaldehyde (Sigma-Aldrich) for 30 min and stained with Alkaline Phosphatase (Fast Blue RR salt, Sigma-Aldrich) according to the manufacturer's instructions.

Alkaline Phosphatase activity colorimetric assay

Cells were seeded in 24-well plates at a density of 4,000 cells/well and grown in presence or absence of nicotinamide or BMP-2 for 24 and 48 h. The ALP activity was measured by colorimetric assay kit (K412-500, Biovision, USA). Briefly, cells were lysed in the ALP assay buffer, the supernatant was collected and incubated with p-nitrophenol phosphate at

25°C for 1 hour. After adding the Stop solution, the absorbance was measured at 405 nm by using Victor 1420 Multilabel counter (Perkin Elmer, Wellesley, MA). The protein content was measured in the cell lysate using the Qubit 2.0 fluorometer. Specific ALP activity was normalized using the total protein concentration.

Immunofluorescent Analysis

Cells were seeded on a glass coverslides in 24-well plate and incubated in presence or absence of BMP-2 and nicotinamide for 0, 0.5, 2, and 4 hours. The cells were washed three times with PBS and fixed with 4% Paraformaldehyde (Sigma-Aldrich) for 30 min and then incubated with 0,5% Tritón X-100 (Sigma-Aldrich, X100-500ML) in PBS for 10 min. After washing 3 times with PBS, the coverslides were incubated in 1% bovine serum albumin (BSA) with 0,1% Tween 20 (Sigma-Aldrich) for 1 hour. Then incubated with primary antibodies and TRITC-conjugated goat anti-rabbit IgG or FITC-conjugated anti-mouse IgG secondary antibodies (Sigma-Aldrich). Images were acquired by the Epifluorescence microscope Nikon Eclipse E600.

Results

Nicotinamide inhibits osteogenic differentiation induced by BMP-2

BMP-2 is a growth factor that is critical in osteoblast differentiation, and has been shown to be capable of inducing the differentiation of pre-myoblast C2C12 cells into osteoblast [15] through the activation of protein type I receptors [16]. Expression of alkaline phosphatase is used as a marker for osteoblast differentiation. BMP-2 has been shown to induce expression of alkaline phosphatase in C2C12 cells with a concomitant increase in enzyme activity [15]. To test whether nicotinamide could exert an inhibitory effect on the osteogenic differentiation of C2C12 cells, we conducted an osteogenic differentiation test in presence of BMP-2 and/or nicotinamide and osteoblast differentiation was analyzed by ALP staining assay. We tested different concentrations of nicotinamide and found that an inhibition of osteoblastic differentiation occurred in a dose dependent manner (Figure 1A top panel). We then used a concentration of 20mM nicotinamide as it did not produce a significant decrease in cell viability (Figure 1A bottom panel) and we observed that at both 24 and 48 hours of treatment, nicotinamide reduced the differentiation of C2C12 cells to osteoblasts under osteogenic conditions (Figure 1B top panel). Treatment with nicotinamide significantly inhibited the alkaline phosphatase activity induced by BMP-2 (Figure 1B bottom panel). Together these results indicate that nicotinamide exerts an inhibitory effect on BMP-2 induced osteoblastic differentiation pathways.

ACVR1 and Runx2 gene expression is independent of nicotinamide treatment

Activin like receptor ACVR1 it is altered in FOP pathology and is responsible for the constitutive activation of the Smad dependent pathway. From previous results, we observed that nicotinamide is able to inhibit *in vitro* osteoblastogenic differentiation (Figure 1). Therefore, it is possible that the osteoblastogenesis inhibition mediated by nicotinamide result from decrease ACVR1 expression or other gene such as Runx2. Runx2 is a master transcription factor regulator of osteoblastogenesis [17]. BMP-2 is necessary for Runx2 activation which produces the induction of osteoblastic phenotype [18]. To examine whether nicotinamide produces a decrease in expression of the ACVR1 receptor and other genes, we tested the relative expression of ACVR1, Runx2 and Fmod at the messenger level using RT-qPCR. As shown in Figure 2 left panel, treatment with nicotinamide only decrease the ACVR1 expression at 16 hours. On the other hand, co-treatment BMP-2 plus nicotinamide does not significantly decrease ACVR1 expression at 16 hours

(Figure 2 A left panel). It can be seen that the expression levels of the ACVR1 receptor remain relatively constant between conditions after 24 and 48 hours of treatment (Figure 2 B and C, left panel). On the other hand, BMP-2 produced a significant increase in the expression of the Fmod gene compared to control and nicotinamide treatment, however the BMP-2 plus nicotinamide co-treatment had no effect on Fmod gene expression compared to BMP-2 treatment at 16 and 24 hours post-treatment (Figure 2 middle panels). Similarly, the master regulatory transcription factor Runx2 significantly increases its expression levels after treatment with BMP-2 at 24 and 48 hours. However, co-treatment with nicotinamide does not produce statistically significant changes in gene expression levels (Figure 2 right panels).

Taken together, these results suggest that the mechanism by which nicotinamide inhibits osteoblastogenesis is independent of *ACVR1* or *Runx2* gene expression.

The Smad-dependent signaling pathway is interrupted by nicotinamide

BMP-2 activity is mediated by activation of Smad-dependent signaling through an increase in phosphorylation and consequent activation of the Smad1/5/8 protein complex. Therefore, we examined activation of Smad1/5/8, which transduce intracellular signals to the nucleus. The BMP-2 mediated activation of Smad signaling pathway can be seen from 0,5 hours and we found that phosphorylation of the Smad1/5/8 complex decreases dramatically at 0.5 hours after initiation of nicotinamide treatment (Figure 3 top panel).

Similarly, the inhibitory effect of nicotinamide can be observed after 2 hours of treatment and to a lesser degree at 4 hours after treatment, whereas the levels of the Smad1 protein remained relatively constant between the treatments.

On the other hand, the immunocytochemistry analysis reveals that after 2 hours of induction with BMP-2 there is a significant increase in the phosphorylation of the Smad 1/5/8 complex, which is observed mainly in a nuclear location. Moreover, co-treatment with nicotinamide produces a decrease in the phosphorylation signal of the Smad complex at both 2 and 4 hours (Figure 3 bottom panel). Taken together these results indicate that nicotinamide can decrease the activation of the Smad signalling pathway induced by BMP-2.

Discussion

Osteogenic differentiation begins when BMP-2 binds to two receptors serine-threonine kinases type I (ACVR1) and type II (BMPRII) forming an oligomeric complex and producing its dimerization. First, BMP-2 is recognized by the type II receptor, which produces phosphorylation of the type I receptor and consequently activates it. Once activated the type I BMP receptor, triggers an intracellular signaling cascade that produces the phosphorylation of the Smad1/ 5/8 proteins, which are associated with Smad4 [19]. The complex formed by Smad proteins translocates to the cell nucleus activating the transcription of Runx2, which is a master regulator that activates the expression of other osteoblasts specific genes, favoring the differentiation and the subsequent synthesis of the bone matrix [18]. The

dysregulation of BMP signaling pathway plays a central role in the process of heterotopic ossification and is the most studied pathway associated with this pathology, although there may also be others pathways involved [20].

In the present study, we found that nicotinamide inhibits the differentiation of C2C12 cells into osteoblasts in a dose-dependent manner. These results agree with the experiments performed by Bäckesjö and collaborators in which cultivating mesenchymal stem cells in the presence of nicotinamide decrease bone differentiation and inhibited mineralization. Additionally, the addition of nicotinamide to mesenchymal mouse stem cells produced a general downregulation in the expression of osteoblast markers, collagen 1 α 1, osteocalcin, Runx2, with a marked downregulation of ALP [21]. Similarly, the assay of ALP activity agrees with the inhibitory effect of nicotinamide on the differentiation of C2C12 cells to osteoblast (Figure 1).

Because nicotinamide inhibits osteoblastogenesis independently of ACVR1 and Runx2 gene expression (Figure 2), we investigated whether nicotinamide had an effect on activation of the Smad 1/5/8 protein complex by phosphorylation. According to our results activation of the Smad-dependent pathway by BMP-2 is diminished in the presence of nicotinamide, which may explain the inhibitory effect on the osteoblast differentiation (Figure 3).

It has been shown that nicotinamide is a potent inhibitor of Sirt1 which is a NAD⁺ dependent deacetylase [22]. This enzyme acts by transferring acetyl groups to their protein targets that include histones and transcription factors [23]. It has been described that Sirt1 is a positive regulator in bone mass formation [24]. Studies have shown that in haploinsufficient mice (Sirt1^{-/+}) there is a significant reduction in bone mass accompanied by a decrease in bone formation and a decrease of osteoblast expression markers genes such ALP, Collagen 1 α 1, Osteocalcin and bone Sialoprotein [25]. Others studies have determined that the overexpression of Sirt1 increases the transcriptional activity of the Runx2 transcription factor, while the silencing and *ex vivo* deletion of Sirt1 produces a decrease in the transcriptional activity and a concomitant decrease of Runx2 downstream target genes. However, the expression of the master osteoblast transcription factor Runx2 was unchanged [26].

With respect to the inhibition mechanism of Smad signaling pathway by nicotinamide, very little information exist. However, the group of Kume et al determined that Smad7 is a protein target of Sirt1. Smad7 is an inhibitory component that prevents phosphorylation of the Smad1/5/8 complex. The researchers showed that Sirt1 deacetylates Smad7 in specific lysine residues. This deacetylation allows the subsequent ubiquitination and degradation of Smad7 via proteasome. They also showed that overexpression of Sirt1 decreases the expression levels of Smad7 and the opposite happens when Sirt1 is silenced by siRNA [27].

Further studies are needed to determine the precise molecular mechanism by which nicotinamide prevents *in vitro* differentiation of mesenchymal cells into osteoblast and to determine if the inhibition of the Smad signaling pathway by nicotinamide occurs through a mechanism that involves Sirt1 and Smad7. New technologies approach Next-Generation sequencing such as RNA-Seq, could be very useful

in helping to elucidate the underlying molecular mechanism and in the discovery of new therapeutic targets for the treatment of heterotopic ossifications.

This finding could be helpful in the clinic for the treatment of diseases related to heterotopic bone formation. The rational use of nicotinamide can be of great utility in pathologies where heterotopic ossification occurs providing a new potential therapeutically approach.

Declarations

Conflicts of interest: The authors declare that they have no competing interest.

Highlights

- BMP-2 induced osteoblastic differentiation is inhibit by nicotinamide
- Nicotinamide inhibits osteoblastic differentiation by a mechanism that is independent of ACVR1 and Runx2 expression.
- Nicotinamide acts by decreasing phosphorylation of Smad 1/5/8 complex.

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Table

Table 1 Oligonucleotide sequences used for quantitative real-time PCR

Target gene	Sequence	Product length	Reference
<i>Runx2</i>	F: 5'- AAA ACC AAG TAG CCA GGT TC- 3' R: 5'- TCA TAC TGG GAT GAG GAA TG- 3'	246	NM_001145920.2
<i>Acvr1</i>	F: 5'- GGA GTT GCT CTC AGG AAG TT-3 R: 5'- GAC ACA CTC CAA CAG GGT TA-3	216	NM_001110204.1
<i>Fmod</i>	F: 5'- CGG TTG TCT CAC AAC AGT CT-3 R: 5'- GCA GCT TGG AGA AGT TCA T-3	217	NM_021355.3
<i>Snrpd3</i>	F: 5'-ATT GGT GTG CCG ATT AAA G-3' R: 5'-TGT CAG GCA AAA TCA GAA AT-3	220	NM_026095.4

Abbreviations : F, forward primer; R, reverse primer.

Figures

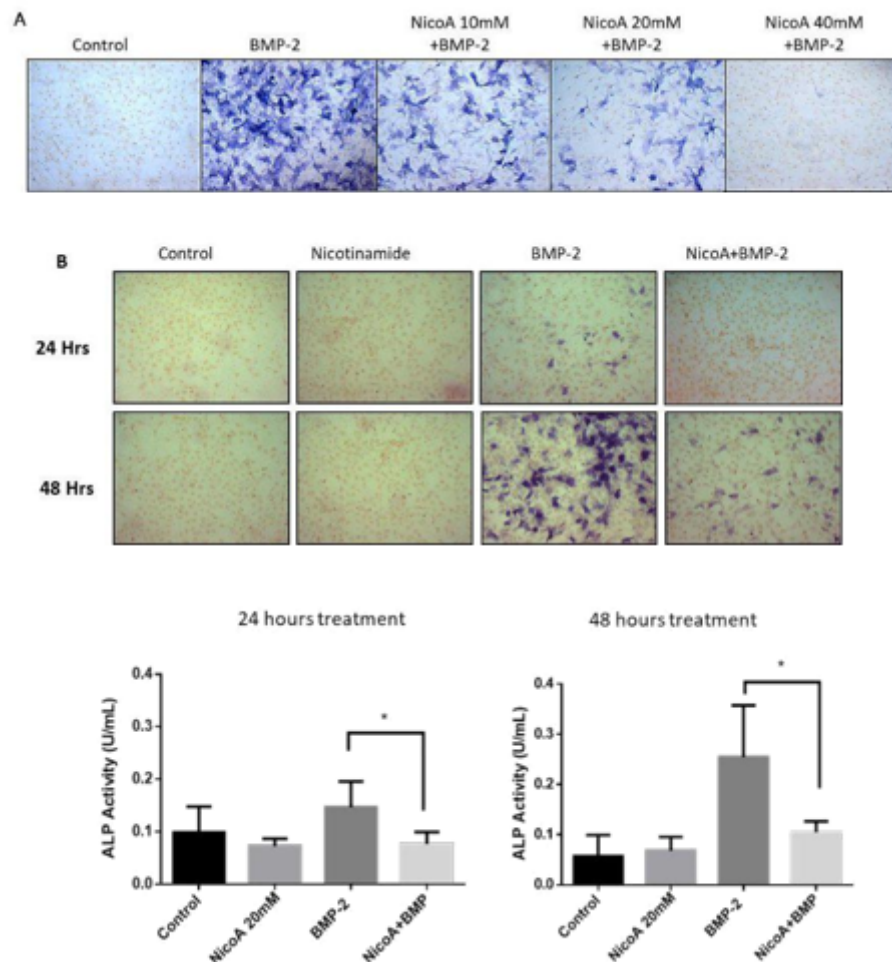


Figure 1

Figure 1A. Osteoblastic differentiation inhibition occurred at different concentrations of nicotinamide in a dose dependent manner. Figure 1B. Both 24 and 48 hours of treatment, nicotinamide reduced the differentiation of C2C12 cells to osteoblasts under osteogenic conditions. Nicotinamide inhibits C2C12 differentiation into osteoblast cells. (A) C2C12 cells were plated at 30,000 cells/well in 24-well plates cultured for 24 and 48 hrs and treated with 500ng/mL of human recombinant BMP-2 and nicotinamide at different concentrations and stained for alkaline phosphatase (ALP) presence (top panel). Cell viability at different concentrations of nicotinamide was evaluated (bottom panel). (B) Evaluation of osteogenic differentiation in the presence or absence of 20 mM nicotinamide and/or 500 ng/mL BMP-2 at different times of treatment (top panel). Alkaline phosphatase activity is shown for the different treatments at 24 and 48 hours as indicated (bottom panel). A p value <0,01 was considered statistically significant. Errors bars represent s.d (n=3)

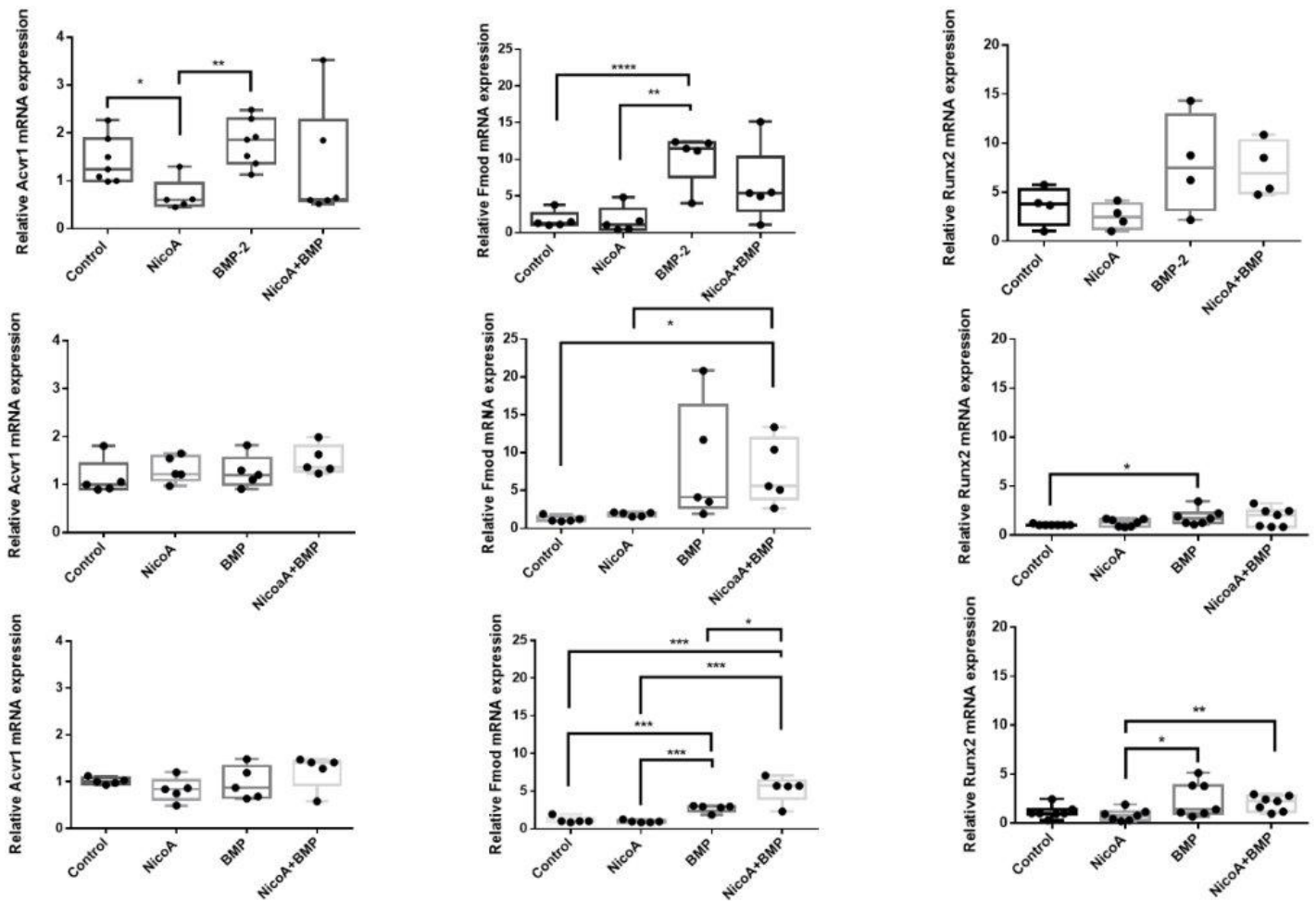


Figure 2

Nicotinamide does not decrease *ACVR1* or *Runx2* gene expression. C2C12 cells were pretreated with nicotinamide 20mM for 4 hours and then co-treated with BMP-2 for 16, 24 and 48 Hrs. Genetic expression for *ACVR1*, *Fmod* and *Runx2* (Left, middle and right columns respectively) at 16, 24 and 48 hours of treatment measured by RT-qPCR is shown in panels A, B and C respectively. A p value * $p < 0.05$ compared with the untreated cells was considered statistically significant. ($n \geq 4$)

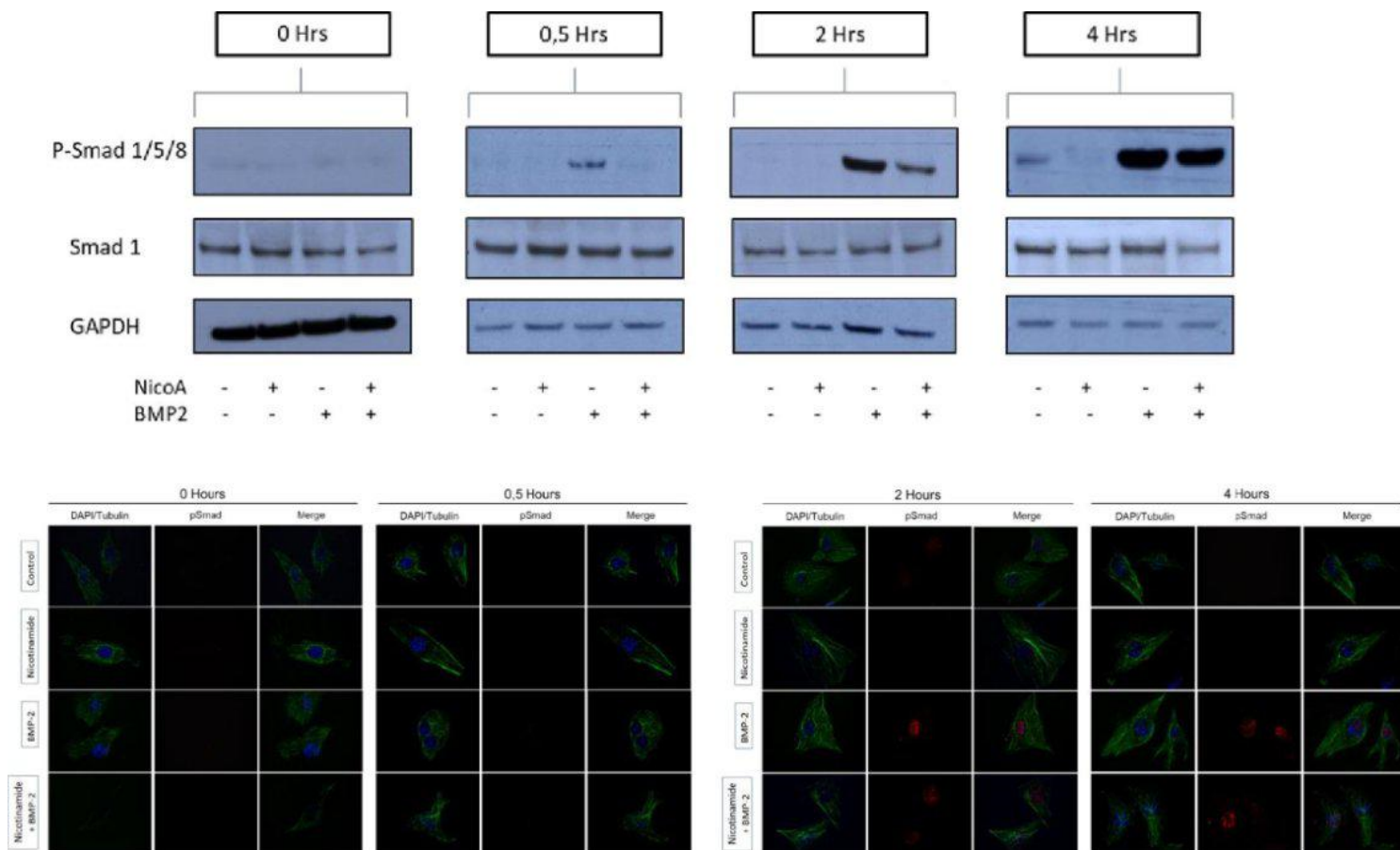


Figure 3

Inhibition of BMP signaling pathway by nicotinamide. C2C12 cells were treated with BMP-2 (500ng/mL) in the presence or absence of nicotinamide (20mM) for 0, 0.5, 2 and 4 hours. Top panel shows a western blot analyses. Bottom panel shows a Immunofluorescent assay, when blue DAPI staining, green Tubulin and red pSmad (higher magnification X100)