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Research Article

Osteoblastic Differentiation's Inhibition Mediated by Nicotinamide

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ABSTRACT

Heterotopic Ossification is a pathological process that is defined as extraskeletal bone formation in both muscle and soft tissue. The most severe condition of HO, a rare genetic form, is the Fibrodysplasia Ossificans Progressive (FOP). Some data suggest that nicotinamide therapy shows major clinical improvement in patients with FOP. This relational analytic study seeks to present the results of molecular findings of the effect of nicotinamide in the differentiation of C2C12 cell line to osteoblasts. To assess this objective, cells were cultured, immunochemistry histologic, and molecular assays were run to analyze the effect of nicotinamide in osteoblastic differentiation in different times of exposure to BMP2 and/or nicotinamide. Nicotinamide inhibits osteoblastogenesis in a dose-dependent way *in vitro* in the C2C12 cell line. It has an inhibitory effect on the phosphorylation of the Smad 1/5/8 complex without a significant difference in osteoblastogenesis classic gene expression. The rational use of nicotinamide could be of great clinical utility as a new preventive therapeutic tool in pathologies where bone formation occurs in extra-skeletal sites.

Keywords: Osteoblastogenesis; Heterotopic ossification; Nicotinamide; SMAD pathway; Osteoblastic differentiation; Endocrinology; Bone targeted therapy

Abbreviations: HO: Heterotopic Ossification; FOP: Fibrodysplasia Ossificans Progressiva; *ACVR1*: Activin A Receptor Type 1; *ALK2*: Activin receptor-Like Kinase 2; SMAD: Family of proteins that function at the cellular level as a second messenger, propagating intracellular signals; BMP-2: Bone Morphogenic Protein 2; BMPR II: Bone Morphogenetic Protein Receptor type II; RNA: Ribonucleic Acid; Mrna: Ribonucleic acid messenger; cDNA: Deoxyribonucleic Acid copy; DNA: Deoxyribonucleic Acid; RT-qPCR: Quantitative Reverse Transcription PCR; *FMOD*: Encoding gene for Fibromodulin; *RUNX2*: Gene that provides instructions for making a protein that is involved in the development and maintenance of the teeth, bones, and cartilage; pSMAD: SMAD phosphorylated protein; NicoA: Nicotinamide

INTRODUCTION

Heterotopic Ossification (HO) is a pathological process which is defined as extraskeletal bone formation in both muscle and soft tissue [1]. Some authors conceptualized it as a flawed process of tissue repair and are trauma and surgery's common complication [2]. HO has two main presentations, the classic nongenetic HO and the rare genetics forms. Numerous clinical situations can develop this pathologic condition such as hip arthroplasties, fractures, infections, thermal injuries, spinal cord, and traumatic brain injuries [3-10].

The osteogenic differentiation process begins when the growth factor BMP-2 binds to two serine-threonine receptor kinases type I (Activin like Receptor 1=*ACVR1*) and type II (BMPR II) forming an oligomeric complex and producing its dimerization [11-12]. First, BMP-2 is recognized by the type II receptor, which produces phosphorylation of the type I receptor and is consequently activated [13]. Once the BMP type I receptor is activated, it triggers an intracellular signaling cascade that produces phosphorylation of Smad 1/5/8 proteins, which once activated form a complex associated with Smad 4 [14-18]. The complex formed by Smad proteins translocate from the cytoplasm to the cell nucleus and activates the transcription of the *RUNX2* gene, which is the master regulator that initiates the activation of the expression of other specific genes in the process of differentiation towards osteoblasts, favoring the differentiation and subsequent synthesis of the bone matrix [19] (Figure 1).

The deregulation of the BMP-mediated signaling pathway plays a fundamental role in the process of heterotopic ossification and is the most studied intracellular signaling pathway associated with this

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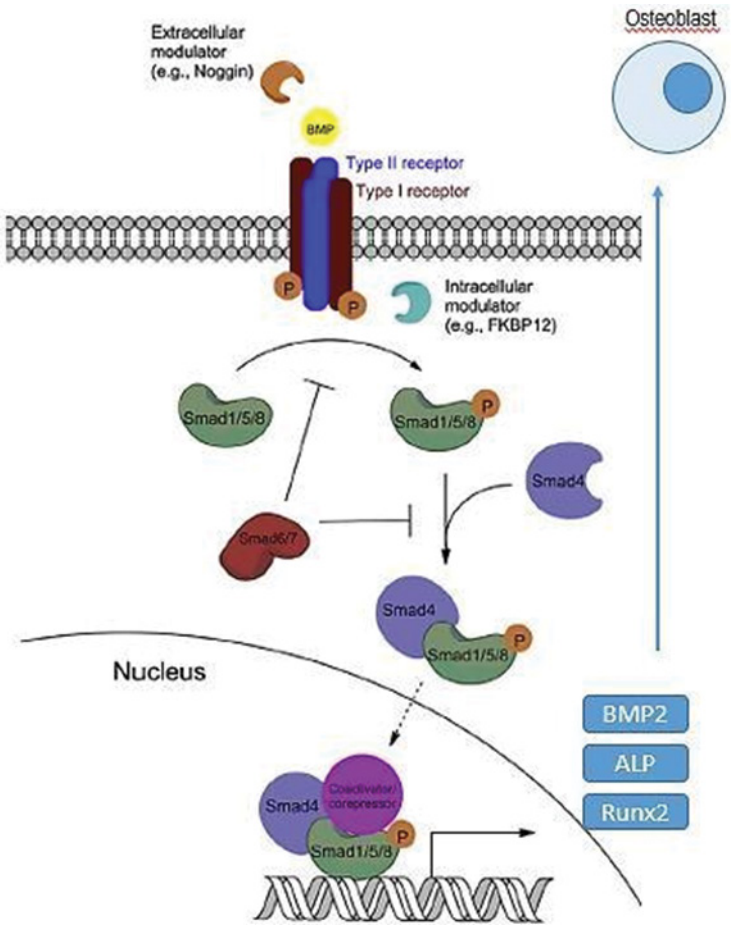

Figure 1: Smad-dependent intracellular signaling pathway activated by BMP. BMP, the bone morphogenic protein is recognized by specific receptors on the mesenchymal stem cell membrane. Receptors phosphorylate Smad proteins, which translocate to the nucleus to activate the expression of genes that eventually differentiate these cells into osteoblasts. (Adapted from Wang et al. [38]).

pathological condition, although there are other pathways involved that may also contribute to the formation of ectopic bone [20].

The effect of various pharmacological compounds on heterotopic ossification acting at different levels of the Smad signaling pathway has been studied. Findings so far about FOP´s pathophysiology are still insufficient and numerous targeted non-interventional and interventional treatments have been reported in the literature, but no consensus in the treatment has been provided yet [21-24].

The most disabling condition of HO known in humans, a rare genetic form, is the Fibrodysplasia Ossificans Progressiva (FOP) [25-27]. FOP is a rare autosomal dominant connective tissue disease, with a prevalence of 1 in 2 million, caused by a point mutation in an *ACVR1* receptor gene, the Activin Receptor-Like Kinase 2 (*ALK2*) [28-29]. *ALK2* mutation triggers constitutive activation of it and consequently a hyperactivation of the SMAD intracellular pathway [30]. This genetic HO is clinically characterized by congenital foot deformities and multiple heterotopic ossifications in fibrous tissue starting with painful soft tissue swellings flare-ups at the age of three or four [31-35]. Until now, no consensus in the treatment has been provided yet.

This study focused on the use of nicotinamide since it has been reported that in patients with FOP, it produces an improvement in their condition, specifically it generates a delay in the appearance of outbreaks of ossification, along with a significant decrease in inflammatory foci [33].

Quevedo and Díaz [33], in their study, included two Chilean children (one of each biological sex) that satisfied the diagnostic criteria for FOP [36]. At the time of diagnosis, ossification was confirmed by radiographs. Both children were asymptomatic until the boy was 1.6 years old and the girl was 8 years old, which was when their respective parents reported the appearance of flare-ups. The neurologic examination was normal. There was no history of arthritis or other musculoskeletal problems in the families of either child.

Various forms of treatment had been employed with both children, such as thalidomide, montelukast, ibuprofen, and prolonged prednisone therapy; but all had failed.

Both children were treated with nicotinamide 150 mg/Kg/day (twice a day), with a maximum dose of 3 g daily (considering it can produce liver toxicity at doses above 3 grams per day) [33].

During the mean follow-up, 2 flare-ups were observed in each one. A rapid decrease in local inflammatory swelling during the week after the appearance of the flare-ups was observed. In contrast, before the nicotinamide treatment, the flare-ups were observed every 3 weeks on average, sometimes with overlapping flare-ups that could continue for as long as 6 weeks. Three of the four flare-ups observed occurred after major traumas. After the first year, the girl discontinued the treatment for three months and the flare-up attacks began again. Radiograph check-ups showed that there was no decrease or increase in the size of previous heterotopic ossifications.

The data suggest that nicotinamide therapy inhibits flare-ups attacks and shows a major clinical improvement in patients with FOP. Our patients received treatment for a mean period of 5 years. No serious adverse events were observed, and the treatment

did not affect their growth. While nicotinamide seemed to have a significant effect in preventing new flare-ups, the authors have no evidence that nicotinamide changed the growth of previous heterotopic ossification [33].

An alternative is nonsteroidal anti-inflammatory medications, cyclo-oxygenase-2 inhibitors, leukotriene inhibitors, and mast cell stabilizers are useful in managing chronic discomfort and ongoing flare-ups. However, nowadays, there is no proven efficacy with any therapy in altering the natural history of the disease [37]. Nicotinamide is a water-soluble vitamin and part of the vitamin B group that can be used with non-diabetic children. Overall, it rarely causes side effects and is considered safe as a food additive.

As the osteogenic differentiation is induced by BMP, a possible explanation for the inhibitory effect of nicotinamide on osteoblastogenesis is that it can act by decreasing the expression of the *ACVR1* gene.

The general objective of this study is to search for relations between the results observed in the case report of two FOP patients treated with high-dose nicotinamide for 5 years and the molecular findings of the effect of nicotinamide in the differentiation of C2C12 cell line to osteoblasts. To achieve this, the following three specific objectives were outlined:

a) Find a molecular pathway through which nicotinamide exerts its action in the C2C12 cell line, as described in the case report, with or without the presence of BMP2

b) Determining if nicotinamide at a molecular level affects the classic osteoblastogenesis gene expression

c) Identify if nicotinamide acts inhibiting the osteoblastogenesis in a dose-dependent or independent way *in vitro* in the C2C12 cell line.

MATERIAL AND METHODS

Reagents and other materials

Dulbecco's Modified Eagle's Medium (DMEM) (11965), Fetal Bovine Serum (FBS), Penicillin-Streptomycin-Glutamine (100X), recombinant BMP-2(PHC7141) were obtained from Gibco (Thermo Fischer Scientific). Bovine Serum Albumin (BSA) (A1595) and Nicotinamide (N0636) were obtained from Sigma-Aldrich. with PBS-1X (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \times 2H_20$; 1.4 mM $KH_2PO_4 \times 2H_20$, adjusting the pH to 7.4) and 100% ethanol were obtained from Merck. TRIzol reagent was provided by Invitrogen. DirectZolTM RNA miniprep kit (#R2050) was from Zymo Research. 0.2 ml PCR tubes were provided by AxygenTM. 0.5 µg oligo (dT), RNase RNasin ® 25 U inhibitor and M-MLV 5X reaction buffer, 0.5 mM dNTPs, and µl reverse transcriptase M-MLV RT were obtained from Promega and nuclease-free water Hyclone™ Hypure Molecular Biology Grade Water from GE-Healthcare.

Cell culture

The preneoplastic C2C12 cell line, which is a subclone from myoblast line established from normal adult C3H mouse leg muscle, was obtained from the European Collection of Authenticated Cell Cultures (ECACC) [39]. The cells were cultured in DMEM, high glucose (11965) medium supplemented with 10% FBS, 29,2 mg/ml L-glutamine, and 100 U/ml Penicillin and 100µg/ml de streptomycin at 37°C in an atmosphere containing 5% CO_2. For osteoblastic differentiation studies, there were $3,5 \times 10^5$ cells seeded in 100 mm culture plates for 24 hours. After that, the culture medium was replaced by a depleted FBS medium, and the latter is then replaced by a BSA 0, 25% for another 24 hours. 24 hours after changing the medium, the cells were treated with recombinant BMP-2 500 ng/ml and/or nicotinamide 10 mM, 20 mM, 40 mM for 16 hours in DMEM, high glucose (11965)

Table 1: List of sequences of primers used for real-time PCR.

Gen Target	Sequence	Product size	Reference
RUNX2	F: 5´-AAA ACC AAG TAG CCA GGT TC-3´ R: 5´-TCA TAC TGG GAT GAG GAA TG-3´	246	NM_001145920.2
ACVR1	F: ´5-GGA GTT GCT CTC AGG AAG TT-3´ R: ´5-GAC ACA CTC CAA CAG GGT TA-3´	216	NM_001110204.1
FMOD	F: ´5-CGG TTG TCT CAC AAC AGT CT-3´ R: ´5-GCA GCT TGG AGA AGT TCA T-3´	217	NM_021355.3

Table 2: Thermal profile of real-time PCR cycles.

No. of cycles	Stage	*RUNX2*	*ACVR1*	*FMOD*
1	Initial denaturation	95°C/10 min	95°C/10 min	95°C/10 min
30	Denaturation	95°C/30 sec	95°C/30 sec	95°C/30 sec
	Alignment	58°C/30 sec	58°C/30 sec	58°C/30 sec
	Extention	72°C/30 sec	72°C/30 sec	72°C/30 sec
1	Final extention	72°C/5 min	72°C/5 min	72°C/5 min

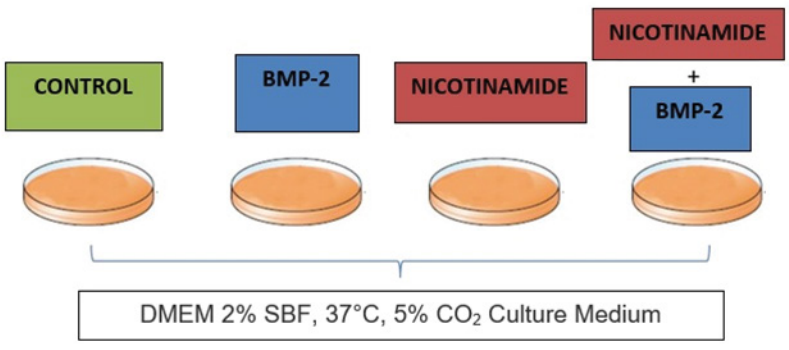

Figure 2: Experimental design of treatments. The mouse C2C12 cell line was used as an *in vitro* model. Four treatments were performed with four independent replicates, which were incubated for 0, 0.5, 2, 4, 16, 24, and 48 hours. The 20 mM nicotinamide concentration is equivalent to that used in clinical trials of patients with FOP.

medium supplemented with 2% FBS at 37°C in an atmosphere containing 5% CO_2. At the same time there were cultured C2C12 cells under the same conditions but without treatment (BMP-2 and/or nicotinamide) which are used as a control group as outlined in Figure 2. The number of replications used was 4.

RNA extraction

Total RNA was isolated from cells grown in the culture plates with DMEM. Before RNA extraction, two washes were performed with PBS1X (137 mM NaCl, Merck; 2.7 mM KCl, Merck; 4.3 mM $Na_2HPO_4 \times 2H_20$, Merck; 1.4 mM $KH_2PO_4 \times 2H_20$, Merck, adjusting the pH to 7.4). For direct lysis of the cells, 1 ml of the TRIzol reagent per 100 mm culture plate (BD Falcon) was used. Then cells were resuspended and incubated for 5 minutes at room temperature, allowing the nucleoprotein complex to dissociate. After that, the cells were centrifuged at 12000 g for 10 minutes at 4°C, discarding the sediment. The obtained supernatant was used for the extraction of total RNA through the DirectZolTM RNA miniprep kit following the supplier's instructions. Briefly, a volume of 100% ethanol was added to the RNA sample in TriZol homogenizing the mixture. Then the sample was transferred to the Zymo-Spin™ IIC Column inserted into a collector tube, centrifuged at 12000 g for 30 seconds at a temperature of 4°C, and the supernatant was discarded. Subsequently, a treatment with DNase I was carried out directly on the column to eliminate possible contaminations with genomic DNA. Finally, the RNA was eluted by adding 50 µl of DNAse/RNAse free water to the column and it was centrifuged at 12000 g for 30 seconds at room temperature. The eluted total RNA sample was stored at-70°C for further analysis and use.

Synthesis of DNA copies (cDNA): cDNA was obtained by using the Reverse Transcription (RT) technique. For this, an M-MLV reverse transcription system (Promega M170B) was used. 2 µg of pre-purified RNA samples were taken and mixed in 0.2 ml PCR tubes with 0.5 µg oligo (dT) and nuclease-free water HycloneTM hypure molecular biology grade water reaching a final volume of 15 µl. Subsequently, the mixture was incubated in a thermocycler (Life Express, model TC-96/G/H) at a temperature of 70°C for 5 minutes to linearize the RNA and then at 4°C for 5 minutes (linearization mix). After the incubation, the tubes were placed on ice to avoid the formation of secondary structures. The RT reaction mixture was prepared in PCR tubes with the following reagents: M-MLV 5X reaction buffer, 0.5 mM dNTPs, RNase RNasin® 25 U inhibitor, 1 µl reverse transcriptase M-MLV RT and nuclease-free water, reaching a final volume of 25 µl. The reaction mixture was subjected to a 42°C PCR cycle for 60 minutes in the previously described thermal cycler. A negative reverse transcription control (non-RT) was performed which did not include the enzyme M-MLV RT.

Real-time PCR

This technique allows us to quantify the PCR amplification products of cDNA samples of genes of interest, this is achieved by amplification and accumulation of double-stranded DNA. The amount of amplified DNA is detected through the SYBR Green I reagent. This reagent is a nucleic acid intercalant, specific for double-stranded DNA, which, when excited at a certain wavelength, emits a fluorescent signal. To perform the real-time PCR, the cDNA produced by the retro-transcription described above was used. The Maxima SYBR Green/ROX qPCR Master Mix (2X) PCR kit (Thermo Fisher K0223) was used. For the PCR reaction, a master mix was prepared by adding the following components: 6.25 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X), Forward 10 µM Splitter (0.375 µl), Reverse 10 µM Splitter (0.375 µl), 2 µl warm cDNA (from the diluted 1:5 stock solution), nuclease-free water to complete a final volume per reaction of 12.5 µl. The list

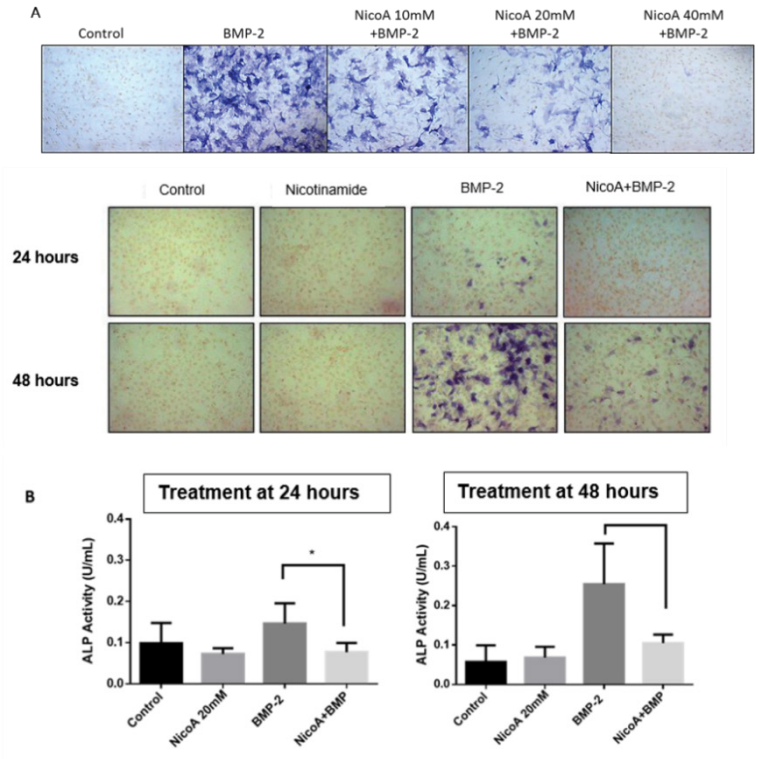

Figure 3: Nicotinamide inhibits osteoblastogenesis *in vitro* of C2C12 cells. A: Osteoblast staining using Naftol AS-MX for the detection of alkaline phosphatase. Treatments were performed at 24 hours and 48 hours. The first row of images is part of the 48 hours exposure assay; B: Bar graph showing alkaline phosphatase activity levels. Standard deviation averages for n=3. A*p<0.01 was considered statistically significant.

of the genes studied, together with the sequence of the starters and their corresponding reference code from the NCBI Gene Bank is shown in Table 1. These genes were chosen because they are part of the classic bone differentiation genes [40]. A non-template control of cDNA (Non-Template control) was also added. The temperatures used in each stage for each set of starters are shown in Table 2. In all tests, the Mx 3000P kit (Stratagene) was used and the results were visualized in the MxPro program. For the analysis of gene expression, the ΔΔ Ct (Delta-Delta Ct) method was used (Schmittgen and Livak 2008) and the statistical analysis was performed with the GraphPad Prism 6 program. The gene-specific starters that were used are the following: *ACVR1*, *FMOD*, and *RUNX2*.

Statistical analysis

The results corresponding to three or more experiments were represented with their standard deviation and submitted to a t-student analysis, which was used to compare two groups. In most of the experiments, each treatment was compared individually with its specific control, and it was determined whether there were significant differences. The $p<0.05$ was used to determine if there were statistically significant differences. GraphPad Prism 6 was used to make graphs and statistical analyses.

RESULTS

Determination of the effect of nicotinamide on osteoblastogenesis *in vitro*

In order to determine if nicotinamide inhibits osteoblast differentiation *in vitro*, C2C12 cells were treated with nicotinamide at a concentration of 20 mM in the presence or absence of the growth factor BMP-2. Previously, Balint et al. [40], determined that, after 16 and 24 post-induction with BMP-2, the bone phenotype is established in C2C12 cells. Therefore, we used exposure periods of 16, 24, and 48 hours to nicotinamide, to determine the possible inhibitory effect of nicotinamide on the bone phenotype. nicotinamide produces a decrease in osteoblast differentiation in a dose-dependent manner (Figure 3A). As seen in Figure 3, a decrease in osteoblast formation is generated in the nicotinamide exposed cells compared to those exposed to BMP-2 alone (Figure 3A). As expected, it is observed that after 48 hours in the presence of BMP-2 there is a greater amount of osteoblasts compared to what is observed after 24 hours.

These results are corroborated by the alkaline phosphatase activity measurement tests (Figure 3B) where significant differences can be observed between the BMP-2 exposed cells compared to the nicotinamide+BMP-2 exposed cells at 24 hours and 48 hours. In both exposure periods, nicotinamide was able to suppress the effect of BMP-2 in the induction of osteoblastogenesis.

Effect of nicotinamide on gene expression in cells undergoing osteoblastic differentiation

To examine whether nicotinamide causes a decrease in the expression of this receptor and in other genes that are expressed in osteoblasts, expression analysis was performed at the mRNA level using RT-qPCR to determine the expression levels of *ACVR1*, *RUNX2*, and *FMOD*. Another probable explanation

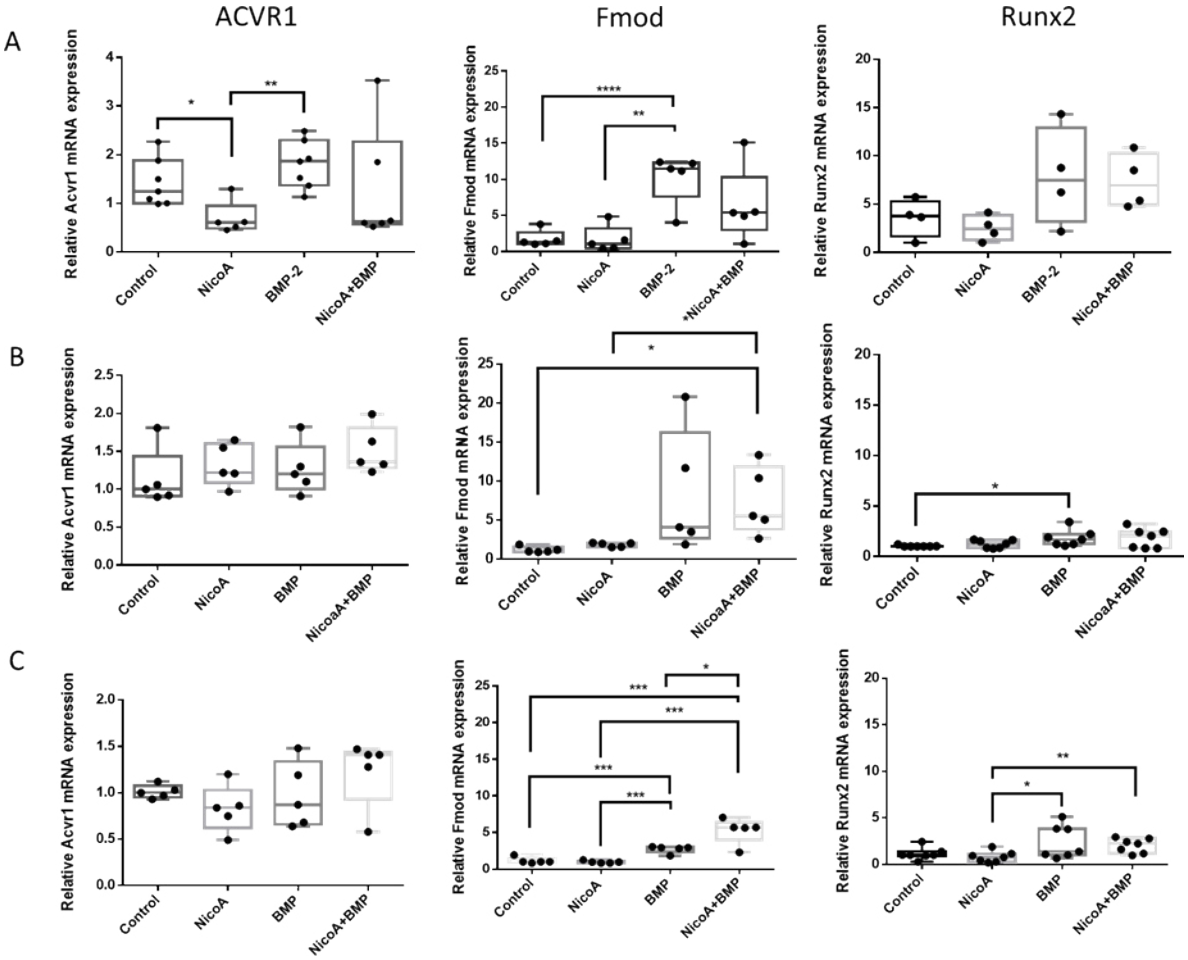

Figure 4: The expression of the *ACVR1* and *RUNX2* genes is independent of nicotinamide treatment. C2C12 cells were exposed to nicotinamide (20 mM) and BMP-2 for 16, 24, and 48 hours. The graphs show the relative gene expression of the mRNAs for *ACVR1*, *RUNX2*, and *FMOD* after 16 hours of treatment; A: for 24 hours; B: 48 hours; C: Quantification was performed using RT-qPCR. A*$p<0.05$ compared to control cells was considered statistically significant ($n \geq 4$).

is that nicotinamide acts by inhibiting the expression of the transcription factor *RUNX2*, which regulates the process of osteogenic differentiation [41]. BMP-2 is necessary for *RUNX2* activation, inducing osteoblastic phenotype [19]. In Figure 4A in the left panel, it is observed that nicotinamide exposure produces a statistically significant reduction in mRNA for *ACVR1*, whereas at 16 hours exposure with BMP-2 alone results in an increase in this mRNA. However, BMP-2 plus nicotinamide exposure did not produce a statistically significant change in *ACVR1* mRNA expression. The *ACVR1* levels remain relatively constant between the different conditions after 24 and 48 hours of exposure (Figure 4B and C, *ACVR1* panel). Furthermore, BMP-2 exposure produced a statistically significant increase in fibromodulin expression compared to control and nicotinamide exposed samples. However, nicotinamide+BMP-2 had no significant effect on gene expression (Figure 4). Similarly, the master regulatory transcription factor for osteoblastic differentiation *RUNX2* significantly increased its expression after exposure to BMP-2 at 24 and 48 hours. However, nicotinamide+BMP-2 did not produce a statistically significant reduction in the expression of this gene (Figure 4).

Effect of nicotinamide on the phosphorylation of the Smad 1/5/8 complex in cells subjected to osteoblastogenic differentiation

In FOP pathology, hyperactivation of the BMP-2-dependent Smad signaling pathway leads to increased phosphorylation and subsequent activation of the Smad 1/5/8 complex [42]. To determine the effect of nicotinamide on the phosphorylation of this complex, the levels of Smad 1/5/8 phosphorylation, which marks the beginning of the activation of the BMP-2 induced osteogenic pathway, were examined by western blot immunoassay and fluorescent immunocytochemistry. The phosphorylation of the Smad 1/5/8 complex can be observed from 0.5 hours of post-exposed samples. As expected, at 0 hours there are no bands that show the activation of this complex, however, after 0.5 hours, a marked band is seen in the presence of the BMP-2 osteoinductor corresponding to pSmad, which shows the activation of the osteogenic pathway through the phosphorylation of this complex (Figure 5). Interestingly, in the nicotinamide, nicotinamide+BMP samples, no development band was observed for pSmad 1/5/8 after 0.5 hours, a situation comparable to that observed in the control. After 2 hours with BMP-2, a marked band is observed in the BMP-2 condition, while in the nicotinamide+BMP condition a decrease in the pSmad 1/5/8 signal is observed. Something similar occurs after 4 hours. Where an increase in the phosphorylation of the Smad 1/5/8 complex is observed in the presence of BMP-2 compared to the joint NicoA+BMP-2 exposed samples. Additionally, it can be observed that there are no changes in the expression of Smad 1 in the different conditions under study.

On the other hand, the immunocytochemistry immunostaining assay using anti pSmad antibodies revealed that after 2 hours of treatment with BMP-2 there is a significant increase in the phosphorylation of the Smad 1/5/8 complex, which is observed in one location mainly nuclear. Additionally, the nicotinamide co-treatment produced an attenuation of the phosphorylation signal of the complex (Figure 6). Similarly, the same effect was observed after 4 hours.

Differential expression analysis

The Cuffdiff program (package that is part of Cufflinks) produces a series of output files, among which the file called gene_exp. diff stands out, which is in a table format and provides information on the differential gene expression indicating whether this expression is significant or not. Table 3 (upper) presents a total number of 326 differentially expressed genes, which is indicated in the last column with the category "yes" (Table 3).

Then the data normalization was carried out to make all the data comparable. As can be observed in Figure 7, on the box plot, the data is normalized and all the conditions are at the same level (log10, fpkm), as well as the observed densities between different conditions practically overlap, so that the data is comparable.

Another analysis carried out consisted of visualizing the relationships between the different conditions in terms of the gene expression fold-change and its statistical significance. For the visualization of the differentially expressed genes with respect to the total of genes, the volcano type graphs were used. As it can be observed, in Figure 8, the comparison between the different conditions shows that there is differential expression according to the fold-change between the different conditions studied.

Principal Component Analysis (PCA) is a multivariate dimensional reduction method of data that can be used to construct dimensional

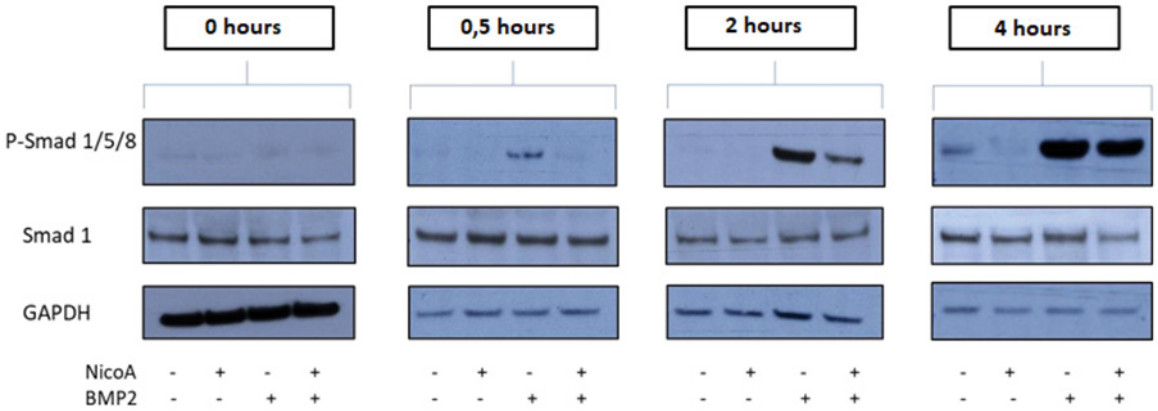

Figure 5: Nicotinamide inhibits the BMP-2 mediated signaling pathway.Western Blot Immunoassay. C2C12 cells were grown in the presence of BMP-2 (500 ng/ml) in the presence or absence of nicotinamide (20 mM) for 0, 0.5, 2, and 4 hours. Activation of the signaling pathway through phosphorylation of Smad proteins can be observed from 0.5 hours after exposure with BMP-2. This figure shows a Western blot assay where the phosphorylation levels of the Smad1/5/8 complex are observed and the levels of Smad 1 protein. GAPDH (constitutive expression gene) was used as a loading control.

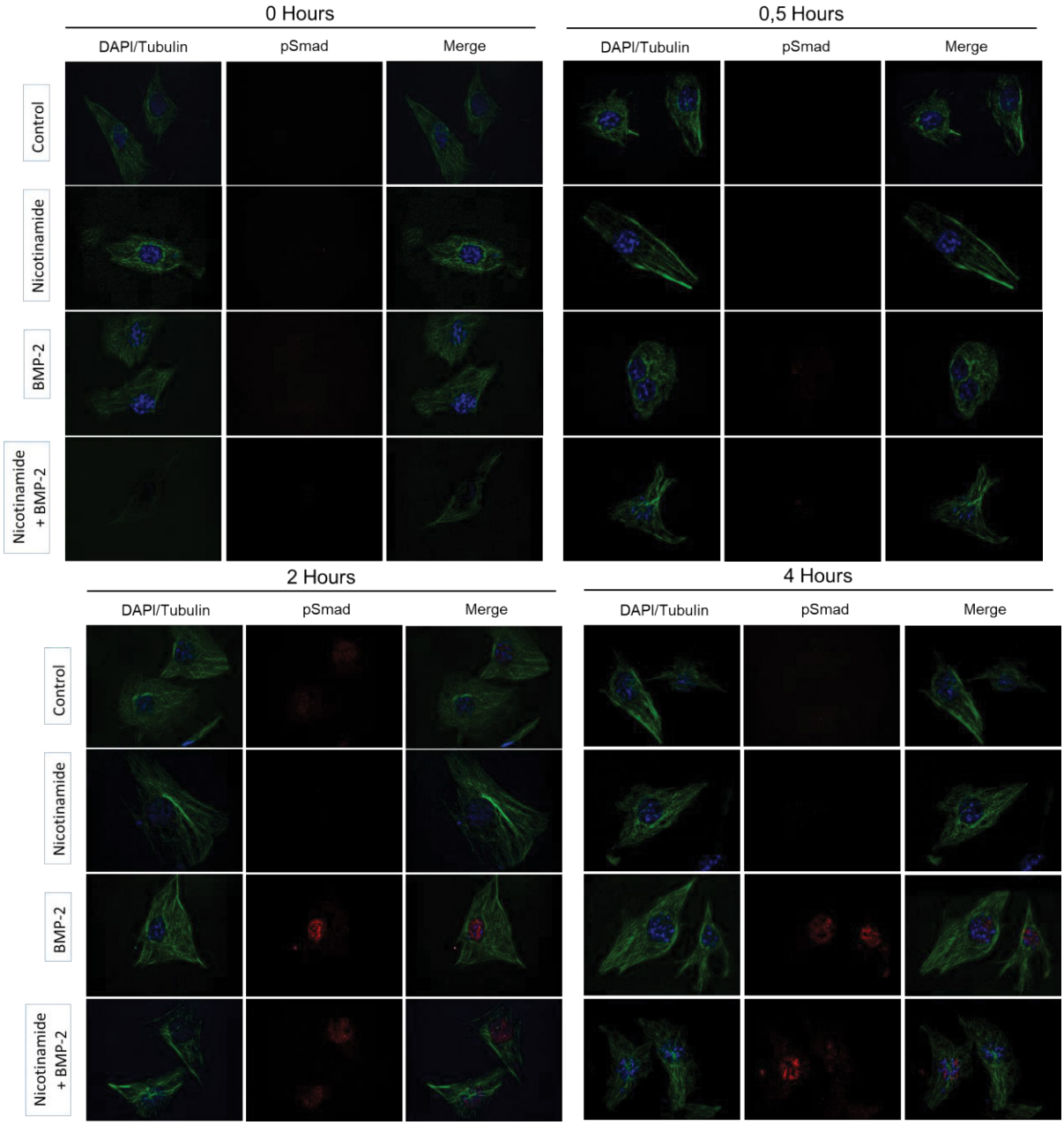

Figure 6: Nicotinamide inhibits the signal of phosphorylation of the Smad 1/5/8 complex. Immunocytochemistry Immunostaining Assay using anti pSmad antibodies. C2C12 cells were grown in the presence of BMP-2 (500 ng/ml) in the presence or absence of nicotinamide (20mM) for 0, 0.5, 2, and 4 hours. This figure shows immunocytochemistry for the phosphorylated Smad 1/5/8 complex (pSmad) labeled with a secondary antibody conjugated with TRITC (red), Tubulin was labeled with secondary anti-mouse antibodies conjugated with FITC (green) and the nucleus was stained with DAPI (blue). The images were acquired by means of an epifluorescence microscope.

Table 3: Genes and transcripts expressed differentially, detected with Cuffdiff.

Column	Differentially expressed genes	Transcripts differentially expressed
Significative difference	326	186
No significative difference	299.275	954.769
Total genes/transcripts differentially expressed	299.598	954.955

representations of expression in each condition. It consists of the use of a mathematical algorithm that reduces the values of thousands of variables in only two components (Main Component 1 and Main Component 2). This allows, for example, to establish relationships between study conditions in addition to allowing evidence of sources of variability within the data and altogether, visually assess similarities and differences between different samples and determine if the samples can be grouped [43]. Another useful type of analysis is the creation of dendrograms, which can provide information on the relationships between different conditions using differential expression data sets. Only genes whose expression is statistically significant are used to represent the relationship between conditions.

In Figure 9A (left) we see the cluster analysis through a dendrogram, where it is seen that there are differences between the different groups under study. Additionally, the control and nicotinamide exposed samples form a cluster with similar elements among them, the nicotinamide+BMP-2 samples are established as a group with similar elements like the nicotinamide-control cluster. On the other hand, exposure to BMP-2 alone forms an isolated cluster and distance from the control and nicotinamide,

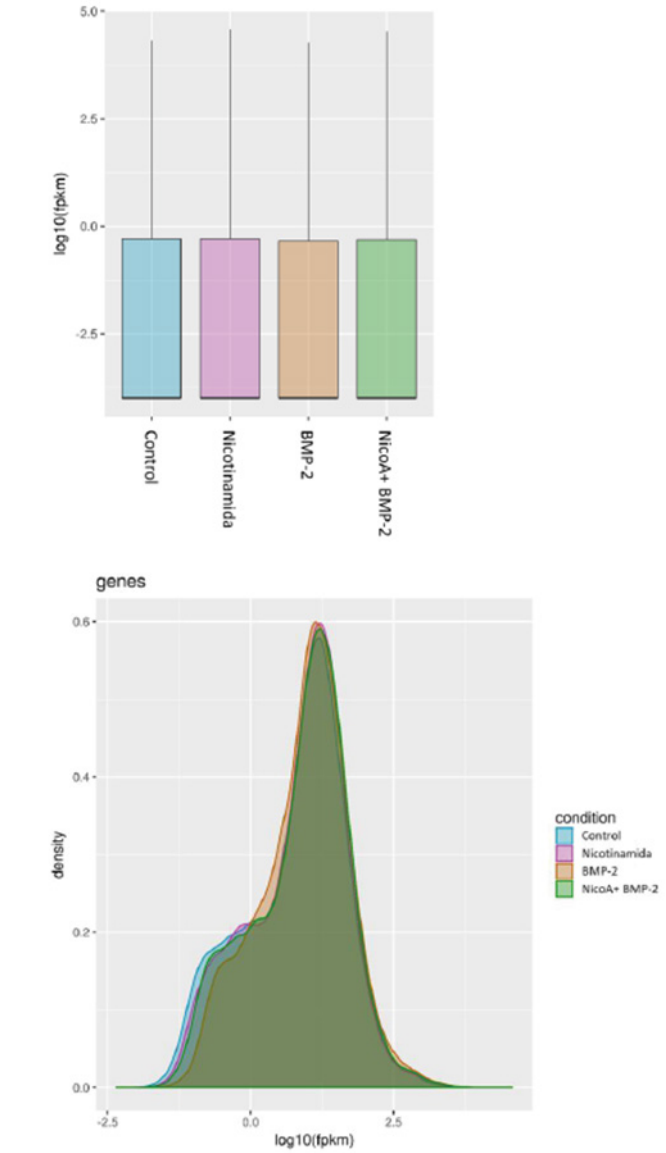

Figure 7: Data normalization. It is observed from the box plot (left) that all conditions are at the same level once normalized. The density graph (right) shows an overlap between the different conditions.

which shows that its elements are the most dissimilar compared to the other conditions under study. Similarly, this is supported by the Principal Component Analysis (PCA) (Figure 9B) where it is first observed that there is no intersection between the different conditions, so they are established in well-differentiated groups. In particular, it can be seen that the BMP-2 condition forms a group well differentiated from the rest of the conditions in the main component 2 and where the formation of a group consisting of the control conditions, nicotinamide, and nicotinamide+BMP-2 is evident.

These results taken together indicate that NicoA+BMP-2 co-exposure gene expression components resemble the control and the nicotinamide sample rather than the BMP-2 sample.

DISCUSSION

In the present study, it was found that nicotinamide inhibits the differentiation of pre-myoblastic C2C12 cells towards osteoblasts in a dose and time-dependent manner as can be seen from the cell differentiation and osteoblast staining assays (Figure 8). These results agree with the experiments carried out by Bäckesjö et al. [44].

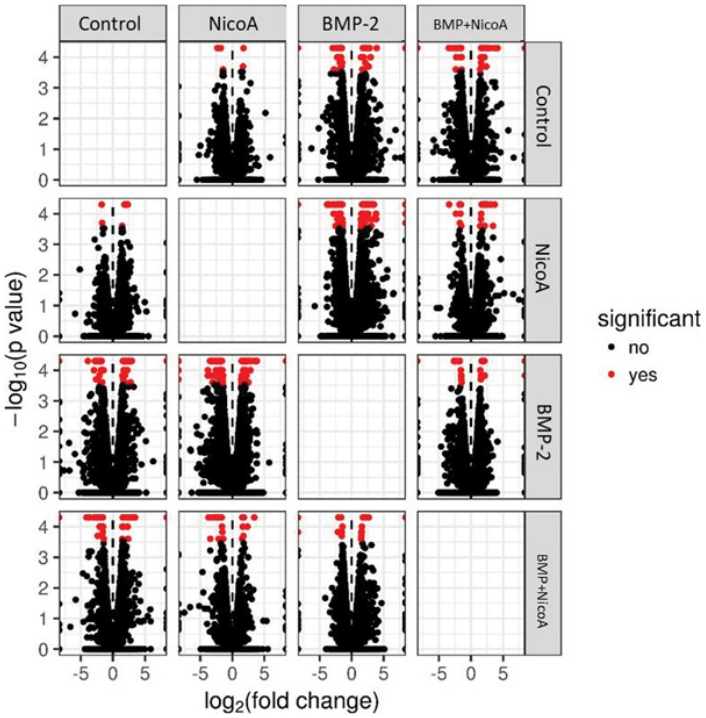

Figure 8: Differential gene expression between different conditions under study. The graph shows the comparative relationships of differential expression between different conditions. Genes expressed statistically significantly are represented as red dots. Genetically expressed genes are represented in black dots.

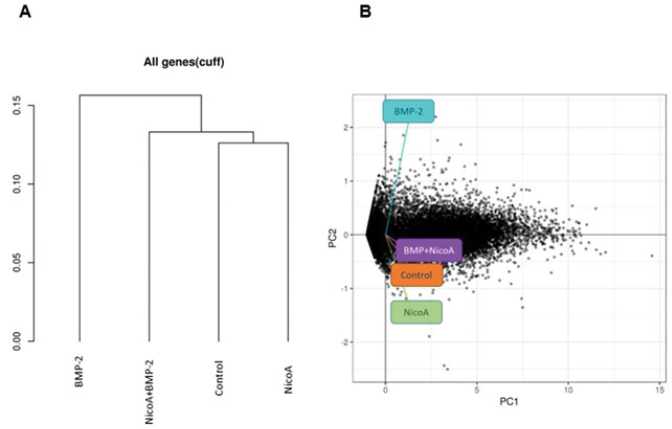

Figure 9: Cluster and principal component analysis. A: shows a dendrogram for the four study conditions; B: represents a graph from the principal component analysis. The graphics were made with the CumeRbund program.

In which they cultured mesenchymal stem cells in the presence of nicotinamide, observing a decrease in osteogenic differentiation in addition to a decrease in mineralization. Additionally, it has been reported that the addition of nicotinamide to mesenchymal stem cells from mice produced a decrease in the expression of osteoblast markers such as collagen 1 alpha-1, osteocalcin, *RUNX2*, with a marked decrease in the expression of alkaline phosphatase ALP [14]. Likewise, the alkaline phosphatase enzyme activity assay supports the results of osteoblast staining, where it is observed that BMP-2 induces strong enzymatic activity, compared to the BMP+nicotinamide, where a statistically significant decrease was observed in enzyme activity, thus counteracting the osteoinductive effect of BMP-2. Taken together, these results indicate that nicotinamide can inhibit osteoclast formation (Figure 8).

On the other hand, the expression of the *RUNX2* transcription factor, which has been described as a master regulator of osteoblastogenesis, is not affected after 16 hours, however, a significant increase in expression could be evidenced both at 24 and 48 hours post-induction with BMP-2 (Figure 9). Similarly, a decrease in *ACVR1* receptor expression was also not evident (Figure 9), so that the inhibition of nicotinamide-mediated osteoblast formation is independent of *RUNX2* and *ACVR1*. These results suggest the participation of other gene products not directly involved with the BMP-dependent Smad pathway. According to various investigations regarding nicotinamide effectors, the possible participation of a deacetylase protein called Sirt1 that is not directly related to the Smad pathway may be indicated. Sirt1 is a NAD+deacetylase enzyme-dependent that is strongly inhibited by nicotinamide [45]. This enzyme works by transferring acetyl groups to their protein targets that include histones and transcription factors [46]. Sirt1 has been reported to be a positive regulator in bone mass formation. Thus, for example, it has been shown that in haploinsufficient mice (Sirt1-/+) a significant reduction in bone mass occurs accompanied by a decrease in bone formation. Additionally, in these studies, there was a significant decrease in alkaline phosphatase activity in mesenchymal cells and a statistically significant decrease in the expression of marker genes such as Collagen1 alpha-1, Osteocalcin, and bone sialoprotein [47]. Researchers have shown that Sirt1 exerts a positive modulating effect on the transcription factor *RUNX2*, which is an indispensable factor for the differentiation of osteoblasts during the bone formation process, both in endochondral ossification and intramembrane [48-50].

Recently, a group of researchers, using mice as a study model, generated a mutation in exon 4 of Sirt1 that corresponds to the sequence that codes for the enzyme's catalytic site. They observed a decrease in both osteoblast differentiation markers and mineralization, evidencing a significant reduction in alkaline phosphatase activity, along with a reduction in the expression levels of Osteocalcin, Osteopontin, and Bone Sialoprotein [48,49]. Additionally, using vectors with the reporter gene luciferase to determine the transcriptional activity of *RUNX2*, the researchers observed that the over-expression of Sirt1 significantly increased the activity of the reporter gene, while, on the other hand, the silencing of Sirt1 by interference RNA caused a decrease in luciferase activity. This indicates that Sirt1 is a positive regulator of the master factor of osteoblastogenesis *RUNX2* [48,49]. The same group of researchers in another published article demonstrated the role of Sirt1 as a positive regulator of bone mass *in vivo*. Knockout mice for Sirt1 showed a significant reduction in bone mass that increases over time. On the other hand, using SRT1720 a Sirt1 agonist, a statistically significant increase in bone mass was observed in the tibia, femur, and spine [49].

Another study showed that, in mesenchymal cells treated with Resveratrol, a Sirt1 activator, the interaction between Sirt1 and *RUNX2* was enhanced and that this interaction decreases when the cells are treated at nicotinamide concentrations of 10 mM and 100 mM. Additionally, using an antisense oligonucleotide for Sirt1 together with nicotinamide, there was an increase in the acetylation state of *RUNX2* and an abrupt decrease in the level of osteocalcin protein, one of the target genes of *RUNX2* indicating that an increase in *RUNX2* acetylation due to Sirt1 inhibition could decrease the transcriptional activity of the transcription factor and therefore inhibit differentiation to osteoblasts [50]. With all the above, the effect in reducing the differentiation of mesenchymal cells to osteoblasts can occur through a mechanism that involves Sirt1, which positively modulates the transcriptional activity of the *RUNX2* transcription factor [48, 49].

Regarding the effect of nicotinamide on the phosphorylation of the Smad 1/5/8 complex dependent on the BMP pathway, we should highlight that this is the first work that determines the decrease in activation of the BMP-dependent signaling pathway (Figure 10). It should be noted that there is very little research that has related this nicotinamide inhibitory effect in the early stages of activation of the Smad complex.

It has been demonstrated by Co-Immunoprecipitation and GST-Pulldown assays that Sirt1 interacts with Smad7, a BMP-dependent Smad pathway inhibitor and that this interaction occurs both *in vivo* and *in vitro* in the Smad7 N-terminal region [18]. This

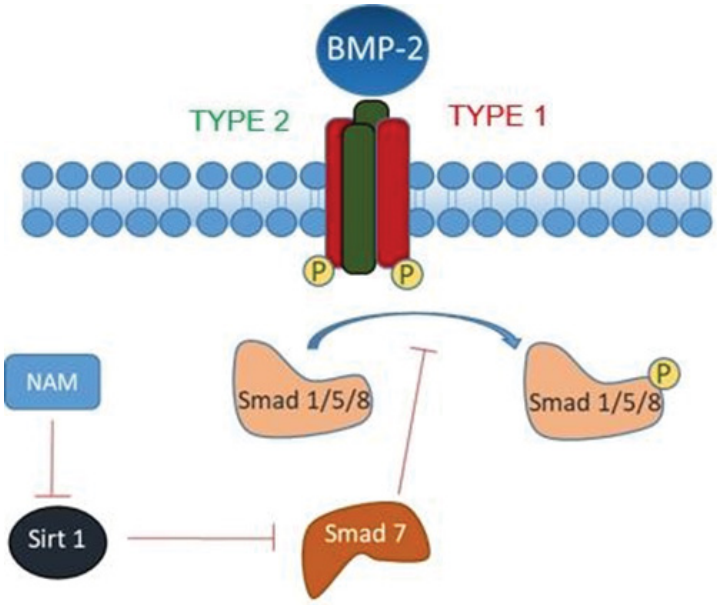

Figure 10: Proposed model of action for the inhibitory effect of nicotinamide on the phosphorylation of the Smad 1/5/8 complex. Nicotinamide is an inhibitor of Sirt 1 that has Smad 7 as one of its protein targets. When Nicotinamide (NAM) is present inside cells, it inhibits Sirt1 and, therefore, Smad7 remains acetylated and can exert its inhibitory effect on the phosphorylation of the Smad 1/5/8 complex.

interaction results in the deacetylation of Lysine residues that protect Smad 7 from ubiquitination and subsequent degradation via the proteasome. Additionally, it was determined that the levels of protein expression of Smad7 were significantly decreased in mesangial cells derived from mice that overexpress Sirt1, but on the other hand, Smad7 protein levels increased against Sirt1 silencing [51]. In this way, Sirt1 is established as a negative modulator of Smad7.

The overall inhibitory effect of nicotinamide on osteogenic differentiation occurs through a mechanism that involves decreased activation of the BMP-2-dependent Smad pathway. With all the above, then a probable inhibition mechanism is proposed that involves nicotinamide as an inhibitor of Sirt1, which in turn negatively regulates Smad7, causing a decrease in the phosphorylation levels of the Smad 1/5/8 complex. However, further studies are required to determine the precise molecular mechanism by which nicotinamide prevents *in vitro* differentiation of mesenchymal cells to osteoblasts by inhibiting activation of the BMP-dependent Smad pathway.

CONCLUSION

Finally, in this study, it was found a molecular pathway through which nicotinamide exerts its action in the C2C12 cell line, as described in the case report, with or without the presence of BMP2. Nicotinamide seems to act inhibiting the osteoblastogenesis in a dose-dependent way *in vitro* in the C2C12 cell line by an inhibitory effect on the phosphorylation of the Smad 1/5/8 complex without a significant difference in osteoblastogenesis classic gene expression. Despite the demonstration of potential benefits of nicotinamide therapy against HO, it would be desirable to perform a multicentric randomized double-blind placebo-controlled therapeutic trial based on homogenous data collection. These subsequent studies can further deepen the role of nicotinamide in the ossification process and can determine the precise molecular mechanism by which it prevents *in vitro* differentiation of mesenchymal cells to osteoblasts, including discovering new therapeutic targets for the preventive treatment of heterotopic ossifications. However, we propose as a model that the inhibition of osteoblast differentiation is through a mechanism that involves Sirt1 and Smad 7. The rational use of nicotinamide could be of great clinical utility as a new preventive therapeutic tool in pathologies where bone formation occurs in extra-skeletal sites.

One of the limitations of this study is that it is only an *in vitro* assay, so further experimentation *in vivo* is desirable. Furthermore, only one signaling pathway was evaluated, though it would be necessary to evaluate other signaling pathways that involve Nicotinamide. Long term effects of Nicotinamide and cytotoxicity should be tested on cell cultures. The study could be replicated in different cell lines. We hope that this study becomes a starting point for new research involving a possible Nicotinamide use as a novel pharmacological treatment for FOP.

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AUTHOR CONTRIBUTION

Fernando Rivas-Valdés: Conceptualization, Methodology, Software, Visualization, Investigation, Software, Validation, Formal analysis Iván Quevedo: Supervision, Writing- Reviewing and Editing, Project administration. Soraya Gutiérrez: Supervision. Gustav A. von Plessing-Pierry: Data curation, Writing- Original draft preparation, Writing-Reviewing, and Editing.

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