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# Hesperetin stimulates differentiation of primary rat osteoblasts involving the BMP signalling pathway

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**Abstract**

Hesperidin found in citrus fruits has been reported to be a promising bioactive compound for maintaining an optimal bone status in ovariectomized rodent models. In this study, we examined the capacity of hesperetin (Hp) to affect the proliferation, differentiation and mineralization of rodent primary osteoblasts. Then, the impact of Hp on signalling pathways known to be implicated in bone formation was explored. We exposed osteoblasts to physiological concentrations of 1 μM Hp (Hp1) and 10 μM Hp (Hp10). Neither proliferation nor mineralization was affected by Hp at either dose during 19 days of exposure. Hp at both doses enhanced differentiation by significantly increasing alkaline phosphatase (ALP) activity from Day 14 of exposure (Day 19: Hp1: +9%, Hp10: +14.8% *vs.* control; $P<.05$). However, Hp did not induce an obvious formation of calcium nodules. The effect of Hp10 on ALP was inhibited by addition of noggin protein, suggesting a possible action of this flavanone through the bone morphogenetic protein (BMP) pathway. Indeed, Hp10 significantly induced (1.2- to 1.4-fold) mRNA expression of genes involved in this signalling pathway (i.e., *BMP2*, *BMP4*, *Runx2* and *Osterix*) after 48 h of exposure. This was strengthened by enhanced phosphorylation of the complex Smad1/5/8. Osteocalcin mRNA level was up-regulated by Hp only at 10 μM (2.2 fold *vs.* control). The same dose of Hp significantly decreased osteopontin (OPN) protein level (50% *vs.* control) after 14 days of culture. Our findings suggest that Hp may regulate osteoblast differentiation through BMP signalling and may influence the mineralization process by modulating OPN expression.


*Keywords:* Hesperetin; Flavonoid; Osteoblast; Differentiation; Bone morphogenetic proteins (BMP)

## 1. Introduction

The maintenance of bone mineral density (BMD) is largely dependent on the coordinated activities of osteoclasts and osteoblasts [1]. Indeed, the imbalance between bone formation and bone resorption occurring in postmenopausal women is the main process implicated in the bone mass decrease observed during aging. Several nutritional factors are implicated in ensuring bone homeostasis. While the most documented among these are calcium and vitamin D, recent animal data suggest that polyphenols and, more precisely, flavonoids could also interact with bone metabolism to improve BMD [2]. Among flavonoids, hesperidin (hesperetin-7-*O*-rutinoside), which belongs to the flavanone subgroup, is found mainly in citrus fruits [3]. When absorbed, hesperidin is hydrolyzed by gut microflora into the aglycone form (Hp) then conjugated into glucuronides, sulfates or sulfoglucuronides [4,5] to reach target cells. In humans, consumption of 1 L of orange juice containing 450 mg of hesperidin led to a circulating plasma concentration of approximately 1 μM Hp [5]. In rodents fed 0.5% hesperidin in the diet, the circulating concentrations of aglycone Hp ranged from 3.5 to 5.5 μM [6,7]. Hp and its metabolites have been shown to exert several biological activities such as antioxidant, anti-inflammatory, analgesic and lipid-lowering effects [8–10]. Regarding bone health, animal studies have shown that hesperidin given at 0.5% in the diet inhibits bone loss in ovariectomized (OVX) mice [6] or rats [7]. Moreover, hesperidin may be responsible for the bone strength modulation observed in male orchidectomized rats following citrus juice consumption [11]. In these different studies, it was shown that hesperidin was able to slow down bone resorption possibly via altered nuclear factor κB (NF-κB) signalling in osteoclast cells [7]. Furthermore, it was hypothesized that hesperidin could not only modulate bone resorption but also affect bone formation by increasing mineral concentrations in the femur of OVX mice [6]. Since hesperidin is also known to lower cholesterol [12], its mechanism of action may be similar to that of statins, which also have a dual action of lowering cholesterol via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and stimulating bone formation. Indeed, postmenopausal women treated

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with statins show increased BMD [13]. Thus, statins are now considered as potential therapeutic agents for patients with osteoporosis [14].

It is well known that osteoblast differentiation is regulated by various pathways such as bone morphogenetic protein (BMP), transforming growth factor β (TGFβ), insulin-like growth factor 1 (IGF1), fibroblast growth factor (FGF), Hedgehog, Wnt (wingless-type MMTV integration site family) and mitogen-activated protein kinase (MAPK) [15–19]. Statins have been shown to enhance osteoblastic BMP production [20,21], as well as induce osteoblast differentiation by modulating other proteins such as alkaline phosphatase (ALP), type I collagen (Coll 1), bone sialoprotein (BSP) and osteocalcin (OCN) [22]. BMPs may activate different signalling cascades corresponding to Smad-dependent and Smad-independent pathways [i.e., extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38/MAPK] [23]. In Smad-dependent pathway, BMP2 and BMP4 bind the receptors, thus inducing the phosphorylation of the Smad1/5/8 complex (R-Smads). R-Smads form a complex with a common partner Smad4 (Co-Smad), which can be translocated into the nucleus to regulate transcription of target genes [24]. At this step, Runx2, a transcription factor known to activate various osteoblast-related genes [15], can interact with R-Smads [25]. Another important transcription factor that may be involved in the same pathway is Osterix [26]. It has been reported that expression of osteoblast markers such as osteonectin, osteopontin (OPN), OCN and BSP was decreased in Osterix-null mutant mice [27]. Concerning Smad-independent pathways such as p38/MAPK signalling [28], it has been reported that BMP2 has an indirect effect on the Fos/Jun family and activating transcription factor-2, which form activator protein 1 (AP-1) transcription factor complexes [29] known to activate osteoblast-related genes [30]. AP-1 and NF-κB are two transcription factors of the MAPK cascade that have been implicated in the regulation of genes involved in bone cell functions. Moreover, it was reported that AP-1 can be induced by both oxidant and antioxidant conditions [31,32].

Little is known about the interaction of polyphenols with these pathways. It has been described that flavonoids may affect the expression of NF-κB and AP-1. Furthermore, many bone-specific genes such as *ALP*, *OPN* and *OCN* have AP-1 sites in their promoters [33]. Indeed, some studies showed that certain phenolic compounds have the ability to regulate AP-1 activity in kidney cells [34] and to increase BMP2 production and phosphorylation of Smad1/5/8, p38 and ERK in osteoblastic cells [35–39]. Regarding flavanones, hesperidin fed to 6- and 24-month-old rats was shown to suppress NF-κB activation through four signal transduction pathways: NF-κB interacting kinase/IkB kinase, ERK, p38 and JNK [40].

The aim of this study was to investigate the effect of Hp on osteoblast functions and to elucidate the cellular and molecular mechanisms involved. Therefore, we assessed the influence of 1 μM (Hp1) and 10 μM Hp (Hp10) on ALP activity, which is the main marker of osteoblast differentiation. We also tested the effect of Hp on mineralization. When considering the molecular impact of Hp, we mainly focused on BMP target genes related to osteoblast functions.

## 2. Materials and methods

### 2.1. Cell culture

Primary osteoblasts were isolated from the calvaria of newborn Wistar rats (INRA, Theix, France) by enzymatic digestion, as described previously [41]. During all experiments, cells were maintained in α-minimal essential medium (α-MEM; GIBCO, Paisley, UK) with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (GIBCO) under conditions of 5% $CO_2$ and 37°C. Cells were seeded on Coll-1-coated (BD Biosciences, Bedford, MA, USA) 96-well plates at a density of 3500 cells/well in 24-well plates ($3\times10^4$ cells/well) or 60-mm Petri dishes ($4\times10^5$ cells/dish) and cultured for 2 days in α-MEM to reach confluence. Cells were then exposed to different conditions: minimal medium ($C^-$), minimal medium containing 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate ($C^+$; optimized medium), and minimal medium supplemented with 1 μM Hp (Hp1) or 10μM Hp (Hp10) (Extrasynthese, Genay, France). Hp was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the medium was 0.1%. Noggin (Sigma, Steinheim, Germany) was added at 100 ng/ml [42] to $C^-$ (Nog) and Hp10 (Hp10+Nog) media. The medium was changed every 2 days.

All cell experiments were performed in triplicate.

### 2.2. Cellular uptake

The cells were treated with a medium supplemented or not with 25 μM Hp for 24 h. Polyphenol was extracted from cells using a solution of 70% methanol and 30% water acidified with 200 mM HCl. Cellular uptake of Hp was determined by high-performance liquid chromatography (HPLC) coul-array detection (Esa, Chelmsford, MA, USA), as previously described [5].

### 2.3. Cell proliferation

Cell proliferation was measured by determining DNA content on Days 0, 5, 9, 14 and 19. Cells were rinsed twice with phosphate-buffered saline (PBS) (Sigma), then incubated with 2 μg/ml bisbenzimide H33342 (Hoechst) in PBS at 37°C in the dark. The total amount of DNA was measured with an FLX800 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA) at wavelengths of 360 nm (excitation) and 460 nm (emission). Fold increase in cell number was calculated relative to the initial cell number on Day 0.

### 2.4. ALP activity measurement

Enzymatic activity of ALP was measured kinetically on Treatment Days 0, 5, 9, 14 and 19 in accordance with the method described by Sabokbar et al. [43], which was adapted to our experimental conditions. Briefly, osteoblasts were rinsed twice with PBS (Sigma) before being frozen at −20°C. The cells were then lysed by freeze–thaw cycle and homogenization into 200 μl of diethanolamine/magnesium chloride hexahydrate buffer (pH 9.8; Sigma). Cell lysates (10 μl) were added to 200 μl of *p*-nitrophenyl phosphate solution (Sigma). Absorbance was measured at 405 nm and 30°C, and every 2 min 30 s for 30 min using an ELX808 microplate reader (Bio-Tek Instruments). ALP activity was expressed as micromoles of *p*-nitrophenol per hour per milligram of protein. Protein measurement was performed in accordance with the method of Bradford [44] using BioRad protein assay (BioRad, Munich, Germany).

### 2.5. Mineralization assay

Osteoblasts seeded in 24-well plates were cultured for 14 days in $C^-$ supplemented with 5 mM β-glycerophosphate and Hp1 or Hp10. Ascorbic acid (25 μg/ml) was only added under $C^+$ condition. On Day 14, calcium nodules were stained with alizarin red S (40 mM) (Sigma), as previously described [45]. For matrix calcification estimate, cells were then incubated in 10% cetylpyridinium chloride solution (Sigma) for 30 min. The absorbance of the released alizarin red S was measured at 562 nm.

### 2.6. Real-time polymerase chain reaction

Upon confluence, cells were exposed to different media ($C^-$, $C^+$, Hp1 or Hp10) for 24 and 48 h. After 24 and 48 h of treatment, total RNA and proteins were isolated using the NucleoSpin RNA/Protein Kit (Macherey-Nagel, Hoerdt, France). Total RNA concentration and purity were measured with a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA integrity was checked using the RNA 6000 Nano Assay kit with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription of RNA was performed using the Ready-To-Go, You-Prime First-Strand Beads Kit (Amersham Biosciences, Piscataway, NJ, USA). The SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa, Shiga, Japan) was used to quantify gene expression by real-time polymerase chain reaction (PCR). The PCR (program: 95°C–30 s; 40 cycles: 95°C–5 s; 60°C–35 s) was performed using a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Target gene expression was normalized to the housekeeping gene *β-actin*. The $2^{-\Delta\Delta C_t}$ method was applied to calculate relative gene expression compared to $C^-$ condition, which corresponds to a value of 1 [46]. The primers used for PCR are listed in Table 1.

### 2.7. Western blot analysis

The concentration of proteins isolated using the NucleoSpin RNA/Protein Kit (Macherey-Nagel) was measured by a BC assay kit (Uptima Interchim, Montluçon, France). Twenty-five micrograms of total protein was subjected to a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to Immobilon-P-PVDF membranes at 100 V for 1 h 45 min. The membranes were blocked in 5% nonfat dry milk in TBS-T (0.5% Tween 20) buffer for 2 h. Blots were incubated with anti-phospho-Smad1/5/8 (Cell Signaling, Beverly, MA, USA) at a 1:1000 dilution for 1 h 30 min at room temperature, washed with TBS-T and then probed with 1:2000 diluted anti-rabbit horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h 30 min at room temperature. The membranes were washed in TBS-T and Tris-buffered saline. Blot signals were detected by enhanced

Table 1
Sequences of real-time PCR primers

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Product size (bp) |
|---|---|---|---|
| *β-Actin* | AGTGTGACGTTGACATCCGTA | GCCAGAGCAGTAATCTCCTTCT | 112 |
| *noggin* | CACTATCTACACATCCGCCCAG | AGCGTCTCGTTCAGATCCTTCT | 110 |
| *BMP2* | GCCAGGTGTCTCCAAGAGACAT | AGCTGGACTTAAGACGCTTCCG | 179 |
| *BMP4* | GACTTCGAGGCGACACTTCT | GCCGGTAAAGATCCCTCATGTA | 100 |
| *Smad1* | CCACAACCCTATTTCGTCGGT | ATCCTGTCTGACTTCTCCGTCC | 102 |
| *Smad5* | TGAACTGAACAACCGTGTCGG | CCTGGTGTTCTCGATGGTTGAG | 151 |
| *Runx2* | CGATCTGAGATTTGTAGGCCG | TCATCAAGCTTCTGTCTGTGCC | 159 |
| *Osterix* | AAGAGGTTCACCCGCTCTGA | TGATGTTTGCTCAAGTGGTCG | 122 |
| *c-jun* | CCTCCCGTCTGGTTGTAGGAAT | CCCTTGCAACACCCTCTTCTTC | 145 |
| *c-fos* | TTCACCCTGCCTCTTCTCAATGAC | GCCTTCAGCTCCATGTTGCTAATG | 82 |
| *ALP* | ACAGCCATCCTGTATGGCAA | GCCTGGTAGTTGTTGTGAGCA | 97 |
| *OPN* | AGCAAGAAACTCTTCCAAGCAA | GTGAGATTCGTCAGATTCATCCG | 129 |
| *OCN* | TATGGCACCACCGTTTAGGG | CTGTGCCGTCCATACTTTCG | 123 |

chemiluminescence (ECL Plus; Amersham GE Healthcare, Buckinghamshire, UK). After stripping in a buffer containing 0.7% β-mercaptoethanol, membranes were labelled with 1:500 diluted anti-Smad1/5/8 and anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and probed with 1:10000 and 1:5000 diluted secondary antibodies, respectively.

### 2.8. BMP2, OPN and OCN concentrations

Osteoblasts seeded in 24-well plates (30,000 cells/well) coated with collagen were grown to confluence and treated. The culture medium was collected after 2, 5 or 14 days of treatment. The concentration of BMP2 protein in the medium was determined using a Quantikine BMP2 enzyme immunoassay (ELISA) (R&D Systems, Minneapolis, MN). OPN and OCN concentrations were measured using a Rat Osteopontin Assay Kit (Immuno-Biological-Laboratories, Gunma, Japan) and a Rat Osteocalcin IRMA Kit (Immunotopics, San Clemente, CA, USA).

### 2.9. Statistical analysis

Results are expressed as mean±S.E.M. ALP activity and cell proliferation on each day were analyzed using parametric one-way analysis of variance, followed by multiple-comparisons Fisher's Exact Test/least significant difference performed in XLSTAT version 7.5.2 (AddinSoft, Paris, France).

Nonparametric test–Wilcoxon signed-rank test (compared to control $C^-$; hypothetical median=1) on GraphPad InStat 3 software (GraphPad, San Diego, CA, USA) was used for statistical analysis of gene expression. $P<.05$ was considered statistically significant.

## 3. Results

In our experimental model, an optimized medium ($C^+$) was used as positive control, and Hp was added to minimal medium ($C^-$) to observe its proper action, and not in synergy with ascorbic acid.

### 3.1. Cellular uptake

The cellular uptake of Hp was tested by incubating cells with 25 μM Hp for 24 h, preparation of cells lysates and analysis by coul array, as described in Materials and Methods.

Examination of the coul-array profile (Fig. 1B) of cell lysates allowed detection of a peak at 17.7 min of retention time, corresponding to Hp when cells were cultivated in a medium supplemented with 25 μM Hp. It was not possible to quantify the exact concentration and cellular uptake of Hp due to insufficient sensitivity of the coul array. However, Hp was not detectable in cell lysates from cells cultivated in $C^-$ medium (Fig. 1A).

### 3.2. Cell proliferation

The impact of Hp on cell proliferation on Days 0, 5, 9, 14 and 19 was assessed by measuring Hoechst incorporation in DNA. As expected, increased proliferation was observed until Day 5 (3.6-fold increase *vs.* Day 0) and remained unchanged after this time under $C^+$ condition. However, a significantly higher proliferation rate was observed in this condition compared to the others (~2.5-fold increase *vs.* Day 0; $P<.01$). Hp did not influence proliferation in these experimental conditions.

### 3.3. Osteoblast differentiation (ALP activity)

Osteoblast differentiation was assessed kinetically by measuring ALP activity in cells treated with Hp for 19 days.

As expected, in osteoblasts treated with optimized medium ($C^+$), ALP activity was significantly increased compared to ALP activity in osteoblasts treated with minimal medium ($C^-$) from Day 5 ($P<.05$) to Day 19 ($P<.001$) (Fig. 2). When Hp was added to $C^-$ at 10 μM, a significant increase in ALP activity on Days 14 and 19 was reported (Hp10: +20.1% on Day 14; +14.8% on Day 19; $P<.001$ *vs.* $C^-$), while only a significant increase was noted on Day 19 with the lower dose (Hp1: +9%; $P<.05$ *vs.* $C^-$) (Fig. 2).

The addition of 100 ng/ml noggin protein into $C^+$ medium was able to diminish ALP activity on each day of treatment when compared to $C^+$. In the medium supplemented with noggin and Hp10, inhibition of ALP activity until Day 14 was observed ($P<.05$ *vs.* Hp10) (Table 2).

### 3.4. Calcium nodule formation

Calcium nodule formation was assessed by measuring the release of alizarin red S after 14 days of cell culture.

As expected, calcium nodules were observed in $C^+$ medium after 14 days of culture (Fig. 3). Indeed, the amount of released alizarin red S was 16 times higher in $C^+$ than in $C^-$. No calcium nodules were detected in $C^-$ or Hp at 1 and 10 μM (Fig. 3).

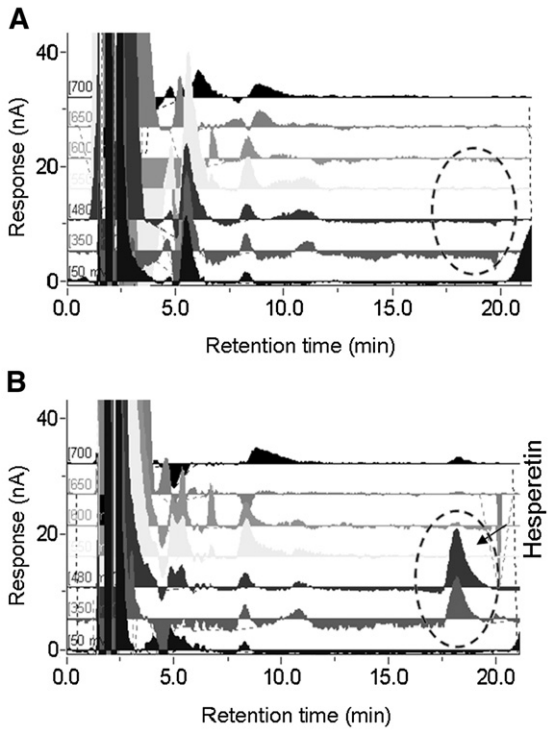


Fig. 1. HPLC coul-array chromatograms of the cellular uptake of Hp. (A) Cell lysate of osteoblasts cultured for 24 h in minimal medium (C−). (B) Cell lysate of primary osteoblasts cultured for 24 h in minimal medium supplemented with 25 μM Hp.

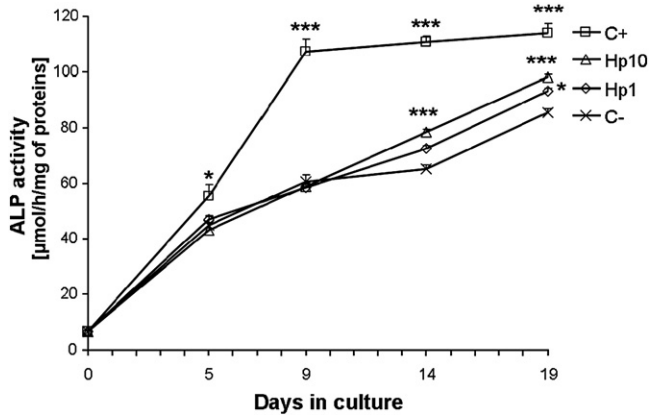


Fig. 2. ALP activity of primary osteoblasts cultured in minimal medium (C−; ×) supplemented with Hp1 (◇) or Hp10 (Δ) or in optimized medium (C+; □) after 0, 5, 9, 14 and 19 days of treatment. Results are expressed as mean±S.E.M. *$P$<.05 and ***$P$<.001 *versus* C−.

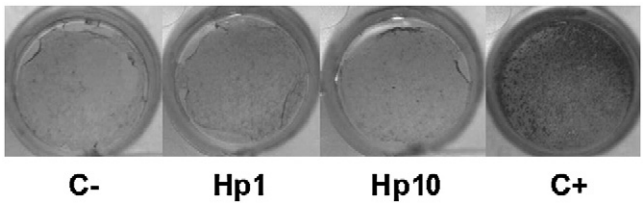


Fig. 3. Mineralized nodules stained with alizarin red S on Day 14 in primary osteoblasts cultured in minimal medium containing 5 mM β-glycerophosphate (C−) supplemented with Hp1 or Hp10 or in optimized medium (C+).

### 3.5. Gene expression (real-time PCR)

To study gene expression, mRNA levels were assessed by real-time PCR after 24 and/or 48 h of treatment. Changes were considered significant when 20% up-regulation (1.2-fold) or down-regulation (1.2-fold) was obtained compared to $C^-$ (value=1).

#### 3.5.1. BMP2, BMP4 and noggin expression

*BMP2* and *BMP4* mRNA levels were up-regulated in $C^+$ medium by 1.5- and 1.9-fold, respectively ($P$<.001 *vs.* $C^-$) and, as expected, the expression of noggin was down-regulated by 3.4-fold in $C^+$ ($P$<.001 *vs.* $C^-$) (Fig. 4). Hp at 10 µM significantly enhanced *BMP2* and *BMP4* expression by 1.22- and 1.43-fold change, respectively ($P$<.01 *vs.* $C^-$). However, the effect of Hp was not dose-dependent, since this increase was higher at Hp1 than at Hp10 for *BMP4* ($P$<.001 *vs.* $C^-$). On the contrary, Hp10 significantly decreased *noggin* mRNA level ($P$<.05 *vs.* $C^-$) (Fig. 4).

#### 3.5.2. Smad1 and Smad5 expression

Expression of both genes was significantly down-regulated in $C^+$ medium ($P$<.001 *vs.* $C^-$). Only *Smad1* transcript level was modified by Hp10 (25% up-regulated; $P$<.001 *vs.* $C^-$) (Fig. 4).

#### 3.5.3. Runx2 and Osterix expression

While *Osterix* expression remained unchanged, *Runx2* expression increased in osteoblasts cultivated in $C^+$ medium ($P$<.001 *vs.* $C^-$). Hp at 10 µM elicited significant up-regulation of *Runx2* (1.37-fold) and *Osterix* (1.43-fold) expression ($P$<.01 *vs.* $C^-$) (Fig. 4).

#### 3.5.4. ALP, OPN and OCN expression

*OCN* mRNA expression was strongly up-regulated (12-fold; $P$<.001 *vs.* $C^-$) in $C^+$ medium, while a decrease in *OPN* expression ($P$<.01 *vs.* $C^-$) was observed. The strongest effects were induced by Hp10 upon the expression of *OPN* (1.66-fold up-regulated), *OCN* (2.2-fold up-regulated) ($P$<.001 *vs.* $C^-$) and *ALP* (1.37-fold up-regulated) ($P$<.01 *vs.* $C^-$) (Fig. 5).

#### 3.5.5. c-jun and c-fos expression

Expression of c-*jun* and c-*fos* in $C^+$ medium was down-regulated at both 24 h (fold change: c-*jun*: 1.38; c-*fos*: 1.36; $P$<.05 *vs.* $C^-$) and 48 h (fold change: c-*jun*: 1.45; c-*fos*: 1.21; $P$<.05 *vs.* $C^-$). Conversely to what was observed at 24 h, expression of both messengers was up-regulated by Hp10 (fold change: c-*jun*: 1.22; c-*fos*: 1.21; $P$<.05 *vs.* $C^-$) (data not shown) after 48 h.

### 3.6. BMP2, OPN and OCN protein levels in culture medium

Under our experimental conditions, BMP2 protein concentration in the medium was not detectable by ELISA after Treatment Days 2 and 5.

In optimized medium ($C^+$), OPN and OCN concentrations were significantly lower compared to those in minimal medium ($C^-$) on Day 14 ($P$<.01). After 14 days of treatment, Hp at 1 or 10 µM was not able to change OCN excretion into medium compared to $C^-$, while OPN concentration was significantly decreased by Hp10 ($P$<.05) (Table 3).

### 3.7. Effect of Hp on phosphorylation of Smad1/5/8

Phosphorylation of Smad proteins was assessed by Western blot analysis of cell lysates from cells incubated for 24 h (Fig. 6A) and 48 h (Fig. 6B). Smad1/5/8 bands represent a nonphosphorylated Smad complex, and p-Smad1/5/8 bands correspond to a phosphorylated complex. The antibodies used in the Western blot analysis were anti-Smad1/5/8 and anti-phospho-Smad1/5/8 antibodies, which can recognize Smad proteins 1, 5 and 8. Because of the specificity of antibodies, as well as the expression of proteins, two bands may be

Table 2
ALP activity of primary osteoblasts cultured in minimal medium (C−) supplemented with Hp10 or 100 ng/ml noggin (Nog) or both Hp10 and 100 ng/ml noggin (Hp10+Nog), or in optimized medium (C+) supplemented with 100 ng/ml noggin (C+ Nog) at baseline and after 5, 9, 14 and 19 days of treatment

| Treatments | Day 0 | Day 5 | Day 9 | Day 14 | Day 19 |
|---|---|---|---|---|---|
| C− | 6.527±0.330 | 45.042±3.318 | 60.855±2.257 | 65.337±1.481$^{\#\#}$ | 85.500±1.604$^{\#\#}$ |
| Hp10 | | 43.246±2.886 | 59.060±1.146 | 78.456±1.356 | 98.121±1.213 |
| Nog | | 37.302±2.728 | 59.613±2.028 | 71.707±2.843 | 89.354±2.102$^{\#\#}$ |
| Hp10+Nog | | 25.965±3.127$^{\#\#}$ | 50.109±1.807$^{\#}$ | 66.392±1.469$^{\#\#}$ | 102.954±1.740$^{\#}$ |
| C+ | | 55,542±4213 | 107,341±4648 | 110,764±2378 | 114,144±3526 |
| C+ Nog | | 43,230±5333 | 100,754±3988 | 102,146±4009 | 94,913±2327 |

Results are expressed as mean±S.E.M.
$^{\#}$ $P$<.05.
$^{\#\#}$ $P$<.001 *versus* Hp10.

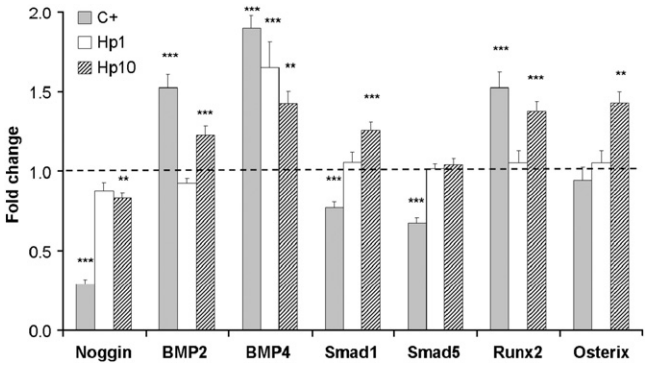


Fig. 4. Gene expression of *noggin, BMP2, BMP4, Smad1, Smad5, Runx2* and *Osterix* in primary osteoblasts cultured in minimal medium supplemented with Hp1 or Hp10 or in optimized medium (C+). Results are presented as fold change compared to C− (dashed line) after 48 h of exposure. Results are expressed as mean±S.E.M. **P*<.05, ***P*<.01 and ****P*<.001 *versus* C−.

visible. Western blot analysis was developed using ECL, which is very difficult to quantify. The observations reported here are qualitative and not quantitative.

At both time points, Smad1/5/8 phosphorylation appeared to be decreased in osteoblasts cultured in $C^+$ medium compared to those cultured in $C^-$ medium (Fig. 6A and B). However, we observed that Hp1 and Hp10 seemed to increase the phosphorylation of Smad1/5/8 (when compared to $C^-$), with this effect being more pronounced after 24 h (Fig. 6A) than after 48 h (Fig. 6B). No difference between the two doses was observed (Hp1 *vs.* Hp10). The protein level of unphosphorylated Smad1/5/8 was not modified whatever the dose of Hp and the time exposure.

## 4. Discussion

It has previously been reported that hesperidin was able to increase BMD in rodents [6,7]. Indeed, hesperidin helps prevent OVX-induced bone loss, as well as improves BMD in sham-operated animals [7]; thus, our hypothesis for its action is that it acts on both resorption and formation. In this study, we focused on the potential bone formation effects and chose the primary rat osteoblast rodent model to study the effect of Hp on proliferation, differentiation, mineralization and associated bone formation signalling pathways

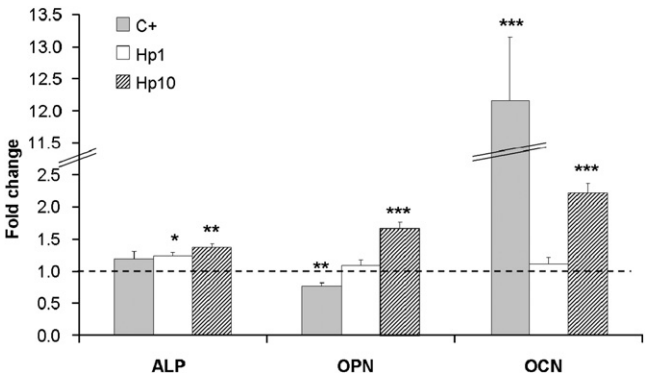


Fig. 5. Gene expression of *ALP, OPN* and *OCN* in primary osteoblasts cultured in minimal medium (C−) supplemented with Hp1 or Hp10 or in optimized medium (C+). Results are presented as fold change compared to C− (dashed line) after 48 h of exposure. Results are expressed as mean±S.E.M. **P*<.05, ***P*<.01 and ****P*<.001 *versus* C−.

Table 3
Concentrations of OPN and OCN in a culture medium of primary osteoblasts exposed to minimal medium containing 5 mM β-glycerophosphate (C−) supplemented with Hp1 or Hp10 or in optimized medium (C+) on Day 14

| Marker | Concentration [ng/ml] | | | |
|---|---|---|---|---|
| | C− | C+ | Hp1 | Hp10 |
| OPN | 54.56±1.15 | 0.714±0.089*** | 39.50±4.42 | 27.42±3.58* |
| OCN | 0.1140±0.0069 | 0.0147±0.0025** | 0.0693±0.0043 | 0.1367±0.0067 |

Results are expressed as mean±S.E.M.
* *P*<.05.
** *P*<.01.
*** *P*<.001 *versus* C−.

such as BMP and MAPK. Certain bone formation agents such as statins have been shown to induce osteoblast differentiation by stimulating the expression of BMP2, ALP, Coll 1, BSP and OCN production in MC3T3-E1 osteoblasts [22]. Since some flavonoids are BMP2 stimulants [47], leading to positive effects on bone formation [20,48–52], our hypothesis was that Hp may influence osteoblast functions potentially through BMP signalling.

### 4.1. Cellular uptake and dose

Cell signalling can be activated by the interaction of a compound with a receptor on the cell surface and/or by an interaction with extracellular proteins [53]. It is also possible that nutrients enter cells then interact with transcription factors, thus influencing target gene expression [54]. Nutrients may enter cells either through transporter-mediated process or by passive diffusion into the cells. In the case of flavonoids, it is known that aglycone forms can cross cell membranes by passive diffusion, but conjugates use active transport through some transmembrane proteins [53,55]. In particular, hesperidin is converted into Hp and conjugates in the body. Despite the fact that the major circulating forms of Hp are conjugates (glucuronides and/or sulfates [4,5]), a possible deconjugation outside the cell, followed by passive diffusion of the resulting aglycone, may occur as it has been shown for other

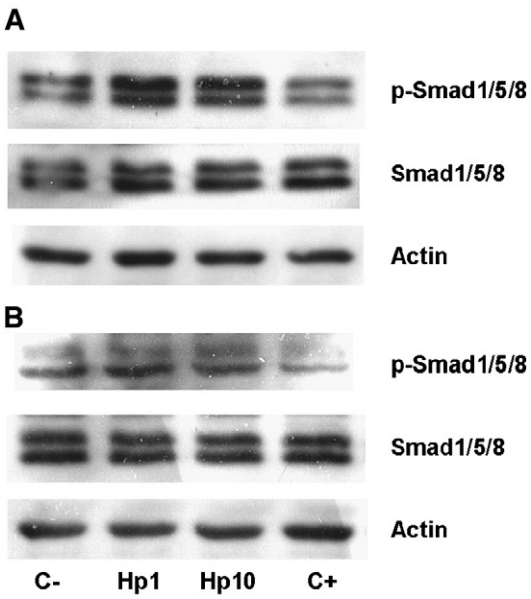


Fig. 6. Phosphorylation of Smad1/5/8 in primary osteoblasts cultured in minimal medium (C−) supplemented with Hp1 or Hp10 or in optimized medium (C+) after 24 h (A) and after 48 h (B) of exposure. p-Smad1/5/8: phosphorylated Smad1/5/8 complex; Smad1/5/8: nonphosphorylated Smad1/5/8 complex.

flavonoids [56]. We thus assessed the cellular uptake of Hp, the aglycone form of hesperidin. In our cellular model, we showed that Hp was taken up into the primary rat osteoblasts (Fig. 1B) although the sensitivity of the coul array was not sufficient to calculate percentage uptake. Our result indicates that Hp entered osteoblasts, as previously described for other cell types such as brain endothelial cell [57], skin fibroblasts [58] and epithelial colorectal adenocarcinoma cells [59], treated also with Hp at supraphysiological doses of 15–30 μM. Thereafter, all the experiments assessing the effect of Hp on osteoblast physiology were carried out in rodent primary osteoblasts with nutritional and physiological Hp concentrations (1 and 10 μM). Indeed, it has been reported that plasma circulating levels of Hp ranged from 3.5 to 5.5 μM in rodents fed hesperidin [6,7].

### 4.2. Choice of model

We chose the primary rat osteoblast model to study the effect of Hp on bone formation and related pathways since this is one of the most commonly used models and allows us to compare our results with previously reported results for flavonoids and statins. In our experimental model, an optimized medium (containing ascorbic acid and β-glycerophosphate) was used as positive control for osteoblast functions. Indeed, ascorbic acid is an important inducer of osteoblast differentiation, and its requirement for both differentiation and mineralization is well known [60,61]. Taking this into consideration, Hp was added to minimal medium to observe its proper action, and not in synergy with ascorbic acid.

### 4.3. Effect of Hp on osteoblast functions

In the present study, Hp, similar to other polyphenols [36,38,62], did not affect osteoblast proliferation, but it stimulated osteoblast differentiation (Fig. 2). These results are consistent with the very recent findings of Choi and Kim [63], which have indicated that Hp from 1 nM to 10 μM significantly increases ALP activity in osteoblastic MC3T3-E1 cells, while proliferation was not affected. Our results showed that Hp at 1 μM (at Day 19) and 10 μM (at Days 14 and 19) increased ALP activity. No significant effect of Hp was observed before Day 14, indicating that the time of polyphenol exposure is essential to observing a significant influence on osteoblast differentiation, as shown by Bu et al. [64] with dried plum phenolics, and/or that differentiation involving many complex mechanisms and signalling pathways takes some time to occur. Moreover, the magnitude of the response in our cell experiments was significant but quite slight. This could be due to the fact that we used nutritional doses and the percentage uptake into the cells might be rather low. Indeed, it has been reported that some polyphenols may modulate ALP activity in a dose-dependant manner [37,38], with the highest dose (20 μM) leading to a stronger stimulation. In our experimental design, we were not able to observe an effect of Hp at 1 and 10 μM on calcium nodule formation (Fig. 3). This does not indicate that Hp failed to influence mineralization. Indeed, expression of OPN and OCN, which are known to be implicated in osteoblast mineralization [65], was significantly up-regulated by Hp at 10 μM (Fig. 5), and OPN protein level was decreased (Table 3). This effect of Hp could be linked to its antioxidant properties. Indeed, redox-responsive elements in the promoter of the *OPN* gene has been reported [66]. Moreover, it is possible that the longer time period of Hp treatment at these doses should be required to influence mineralization up to crystallization. Moreover, the lack of ascorbic acid could also explain the absence of calcium nodules [60]. We concluded that Hp alone was not sufficient to induce nodule formation in our cell culture model. Actually, recent studies have shown that some flavonoids, when combined with ascorbic acids such as epigallocatechin-3-gallate [67] and icaritin [68], were able to stimulate calcium nodule formation in osteoblastic cells. Moreover,

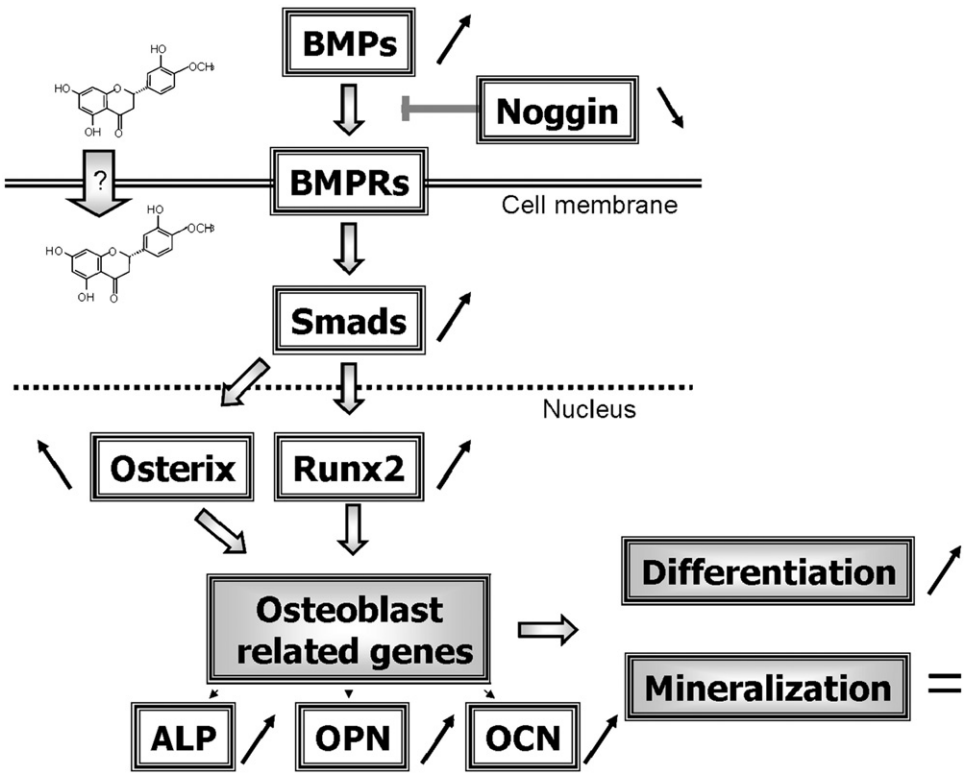


Fig. 7. BMP pathway involved in Hp effect on osteoblast functions.

the same observation was reported for statins [22,69]. However, in these experiments, the effect of compounds alone on mineralization was not tested.

### 4.4. Possible signalling pathways involved

For all the genes evaluated, the level of mRNA responds to different doses of Hp (Figs. 4 and 5). Globally, Hp at 10 μM was efficient, while no effect at 1 μM was observed, except for the *BMP4* and *ALP* genes. All the mRNA levels were analyzed after 48 h of exposure, as commonly reported in the literature for genes involved in differentiation [39,61,70]. However, genes involved in the osteoblast phenotype, including *ALP*, *OPN* and *OCN*, are expressed at different times of differentiation [71]; when possible, confirmation of an effect on protein levels was also assessed.

The BMP pathway can be regulated by a negative feedback loop. Noggin is one of the osteoblast-secreted proteins that can limit the level of BMP signals through complexation with BMPs and prevention of their receptor binding [72]. In our experiment, noggin addition to $C^-$ medium had no effect on ALP activity. However, when added to Hp medium, there was an initial decrease in ALP activity (up to Day 14), suggesting inhibition of BMP signalling in the presence of noggin (Table 2). Other authors have demonstrated the same effect of noggin on phenolic compounds such as myricetin-, piceatannol- and coumarins-treated osteoblasts [36–38]. Surprisingly, at later time points (Day 19) following prolonged noggin treatment in Hp-supplemented medium, ALP activity was no longer suppressed; rather, an increase was observed when compared to Hp alone (Table 2). This could be due to the fact that noggin was added during the whole experiment until Day 19, contrary to other studies where noggin effect was only studied after 2 days of treatment [36–38]. These results suggest that noggin inhibited the early cascade of BMP2/BMP4-dependent events but that, at later times, Hp was able to overcome this inhibition by inducing ALP via other signalling pathways involved in differentiation.

We observed that both Hp and ascorbic acid can stimulate BMP2 and BMP4 expression, as well as down-regulate noggin expression, with ascorbic acid being more efficient (Fig. 4). This indicates that both molecules may have some mechanisms of action in common, but also some specifics as the levels of efficacy are quite different.

Hp also enhanced Smad1 mRNA level (Fig. 4) and was able to increase Smad1/5/8 phosphorylation (Fig. 6), suggesting that Hp could act through a BMP-induced Smad-dependent manner, as previously described for myricetin [38]. Moreover, Hp increased the expression of two main transcription factors (Runx2 and Osterix) (Fig. 4) related to osteoblasts [73] and involved in BMP signalling [15].

These results support our hypothesis that one of the pathways that Hp may activate during osteoblast differentiation could be BMP signalling.

On the other hand, our results concerning OPN and also c-*jun* and c-*fos* gene expression suggest that other pathways such as Wnt, Hedgehog and MAPK, which are known to be implicated in osteoblast differentiation [15–17], should also be considered in the potential mechanism of action of Hp in stimulating bone formation/differentiation. Indeed, Hp after 24 or 48 h of treatment slightly modulated the expression of c-*jun* and c-*fos*, which are the part of the dimeric transcription factor complex AP-1 known as an activator of osteoblast proliferation and differentiation-related genes [33]. Actually, many bone-specific genes such as *ALP*, *OCN* and *Coll 1* have AP-1 sites in the promoters [33]. Our results indicate that Hp, by modulating c-*jun* and c-*fos* expression, could alter AP-1 activity, as previously shown for other flavonoids [34]. However, the real-time PCR results observed for c-*jun* and c-*fos* expression were very small differences, and confirmatory results with effects on the protein/transcription factor changes would be required.

These results indicate that Hp may act on osteoblasts not only through BMP signalling but also through MAPK, as previously described by Kim et al. [40] in kidneys.

## 5. Conclusion

Taken together, our findings suggest that Hp stimulates differentiation in primary rat osteoblast cells involving Smad-dependent BMP signalling pathways (Fig. 7), but not exclusively, as other pathways such as MAPKs are also affected. Thus, Hp seems to be an interesting nutritional compound to consider for further studies with respect to its bone-modulating effect both *in vitro* and *in vivo*.

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