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Ghrelin uses the GHS-R1a/Gi/cAMP pathway and induces differentiation only in mature osteoblasts. This ghrelin pathway is impaired in AIS patients

Ronan Barre [a,b], Nicolas Beton [b,c], Aurélie Batut [b,c], Frank Accabled [c,d], Jerome Sales de Gauzy [c,d], Françoise Auriol [b,e], Sanaa Eddiry [b,e], Maithe Tauber [b,c,e], Sara Laurencin [b,f], Jean Pierre Salles [b,c,e,1], Isabelle Gennero [b,c,g,*,1]

[a] Oral Biology Department, Faculty of Odontology, Paul Sabatier Toulouse III University, France
[b] INSERM, UMR1043, Centre de Physiopathologie de Toulouse Purpan, CHU Toulouse, France
[c] Faculty of Medicine, Paul Sabatier Toulouse III University, France
[d] Orthopedic Department, Children Hospital, CHU Toulouse, France
[e] Endocrinology and Bone Pathologies Department, Children Hospital, CHU Toulouse, France
[f] Periodontology Department, Faculty of Odontology, Paul Sabatier Toulouse III University, France
[g] Clinical Biochemistry Department, Federative Institute of Biology, CHU Toulouse, France

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ABSTRACT

We have examined the Acylated Ghrelin (AG)/Gi pathway in different human osteoblastic cell lines. We have found that: 1) AG induces differentiation/mineralization only in mature osteoblasts; 2) the expression of GHS-R1a increases up to the mature cell stage, 3) the action is mediated via the GHS-R/Gi/cAMP pathway only in mature osteoblasts, and 4) osteoblastic cells from adolescent idiopathic scoliosis (AIS) are resistant to the AG/Gi/cAMP pathway. Altogether, these results suggested that AG uses the GHS-R1a/Gi/cAMP pathway to induce differentiation in mature osteoblasts only. This pathway is impaired in AIS osteoblasts. Understanding AG-specific pathways involved in normal and pathological osteoblasts may be useful for developing new treatments for pathologies such as AIS or osteoporosis.

1. Introduction

Ghrelin is a 28 amino acid acylated peptide (AG) synthesized and secreted mainly by neuroendocrine X/A-like stomach cells [1]. It has a variety of functions in the brain and peripheral tissues [2–5]. Several studies have proposed the involvement of AG in bone growth with potential pathological consequences. Serum ghrelin level may also serve as a marker of bone activity [6–8].

In its specific acylated form, Ghrelin activates the growth hormone secretagogue receptor-1a (GHS-R1a), a G protein coupled receptor (GPCR) identified as the first Ghrelin receptor, although other unidentified GHS-Rs likely exist [1]. Unacylated ghrelin (UAG) represents 90% of the circulating form and has important endocrine effects through still unknown receptors [2,9–11]. Genetically modified mice models have suggested distinct impacts of AG, UAG, and GHS-R1a on *in vivo* trabecular and cortical bone metabolism as well as on *in vitro* osteoblast and osteoclast proliferation or differentiation [11–17]. To date, known GHS-R1a downstream signaling pathways in somatotropic pituitary cells involve Gαq11-protein and phospholipase C activation to release growth hormone [2]. Other mechanisms were reported in various cell types [18]: the PKA/CREB pathway, the Gi-mediated pathway, or heterodimerizations of GHS-R1a with other GPCRs leading to Gi or Gs activation. Ghrelin signaling in osteoblasts remains controversial and the Gs/Gi pathway has not been investigated clearly. Although the controversial expression of GHS-R1a has been suggested in human primary osteoblasts [12], many articles failed to distinguish GHSR-1a from the truncated, inactive form GHS-

R1b. Thus, in many cases, the name GHS-R is used. n bone physiopathology, the current view is that sustained overexpression of the Gs pathway in osteoblasts increases trabecular bone formation with the

* Corresponding author. INSERM UMR 1043, Centre de Physiopathologie de Toulouse Purpan, 31059, Toulouse Cedex 9, France.
E-mail addresses: ronan.barre@gmail.com (R. Barre), nicolas.beton@inserm.fr (N. Beton), aurelie.batut@inserm.fr (A. Batut), accabled.f@chu-toulouse.fr (F. Accabled), salesdegauzy.j@chu-toulouse.fr (J. Sales de Gauzy), auriol.f@chu-toulouse.fr (F. Auriol), eddiry.s@chu-toulouse.fr (S. Eddiry), tauber.m@chu-toulouse.fr (M. Tauber), laurencin.s@chu-toulouse.fr (S. Laurencin), salles.jp@chu-toulouse.fr (J.P. Salles), isabelle.gennero@inserm.fr (I. Gennero).
[1] These authors contributed equally to the work.

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production of immature bone [19–21], while Gs inhibition decreases trabecular bone volume, and number of mature osteoblasts [22]. Conversely, overexpression of the Gi pathway decreases bone mass [23], while its suppression enhances cortical and trabecular bone formation [24–26]. The parathyroid hormone/parathyroid hormone-related receptor-1 (PTHR1) is essential for activating the Gs pathway in osteoblasts [27], and nowadays, recombinant 1–34 PTH biotherapy is used in the treatment of osteoporosis. However, only discontinuous administration elicits bone formation and osteoblast survival, while continuous administration leads to bone resorption [27]. Gi-dependent responses in osteoblastic cells depend on activation of various GPCRs such as apelin, melatonin, cannabinoid, sphingosine-1-phosphate, or lysophosphatidic acid receptors. We have recently reported that LPA1 invalidation is responsible for osteoporosis [28–30] and the Gi/melatonin pathway has been shown to be dysregulated in mature osteoblasts from patients with adolescent idiopathic scoliosis (AIS) [30,31]. We also observed high serum levels of ghrelin in AIS [8], which were shown to predict the curve progression of the disease [32], suggesting a resistance to the ghrelin pathway.

Here, we aimed to clarify the effect of AG on osteoblastic cells differentiation and the intracellular pathways used. The results suggest that AG elicits a GHS-R1a/Gi/cAMP pathway only in differentiated osteoblasts. In addition, we observed resistance to this AG/Gi pathway in osteoblasts of AIS patients. Tuning of the Gi pathway by ghrelin and GHS-R1a may be needed for optimal differentiation of bone cells with potential consequences in AIS physiopathology.

2. Materials and methods

2.1. Cell culture

Primary human osteoblasts and two human osteosarcoma cell lines (Saos-2 and MG63) were cultured in a basic medium (BM) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin (10,000 IU/ml and 10,000 g/ml, respectively) (reagents from Gibco, Invitrogen Corporation). Cells were seeded in 6- or 12-well plates at a density of 10^4 cells/cm^2 and 2×10^4 cells/cm^2 for MG63 and Saos-2, respectively. MG63 cells were cultured for 3 weeks in osteogenic medium (OM) containing 10 nM dexamethasone and 100 μM ascorbic acid, and 5% (v/v) FCS in DMEM to obtain differentiated MG63 cells (dMG63). For the mineralization assay, the medium was supplemented with 10 mM inorganic phosphate. All cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Primary human osteoblasts were obtained from vertebrae bone samples taken from AIS and non AIS scoliotic patients during surgery in the Pediatric Orthopedic Unit of the University Hospital Centre of Toulouse (France). Subjects were recruited from a clinical research program (Clinical trial ID-RCB:2009-A011011-56, granted 01/19/2010) approved by our institutional review board (the CPP Toulouse 1 ethics committee), and after informed consent from the parents of all subjects [8].

2.2. Alkaline phosphatase (ALP) activity, DNA content, and mineralization assay

Cells were washed in PBS, scraped with 0.1% NP40 (v/v) in PBS, and sonicated for 10 s. ALP activity was assayed in cell homogenates as already described [13], except that the experimental values were normalized with the DNA content of cells measured using the Picogreen® assay according to the manufacturer's instructions (Invitrogen Molecular Probes). ALP activity was expressed as nmol *p*-nitrophenol. min^{-1}. ng^{-1} DNA. Calcium deposits from cultured cells were stained with 40 mM Alizarin Red (AR) solution, pH = 4.2, as described previously [29].

2.3. Intracellular cyclic AMP assay

Cell lines were cultured for 24 h in basic medium and further incubated without serum for 24 h. Cells were stimulated with 10^{-5} M forskolin or vehicle (DMSO 0,1% v/v), with or without AG, for 10 min (PolyPeptide Group, Strasbourg, France). When used, 1 μM pertussis toxin (PTX) and 1 μM GHRP6 [D-Lys] were added 12 h and 2 h before the experiments, respectively. The intracellular cAMP content of the cell was measured using an enzyme immunoassay according to the manufacturer's instructions (Amersham-Pharmacia Biosciences).

2.4. Western blot

Cell extracts were placed in a lysis buffer (25 mM Tris, pH 7.4, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 0.5% Triton X-100, 20 mM NaF, 2 mM Na3VO4, and protease inhibitors) and cleared by centrifugation. Aliquots were diluted in a Laemmli sample buffer, boiled, and processed for immunoblotting using a standard procedure. The membranes were incubated overnight at 4 °C, with the primary antibody in PBS containing 0.1% Tween-20. Polyclonal anti-GHS-R1a antibody was purchased from Alpha Diagnostic International (San Antonio, TX) and polyclonal anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunopositive bands were detected by chemiluminescence using ECL reagent (Chemiluminescent Peroxidase Substrate-3, Sigma) and exposed to a radiographic film (Kodak Biomax Light film).

2.5. Real-time quantitative reverse transcription PCR

Total RNA was isolated using Trizol® reagent and concentration and purity were determined by reading the absorbance at 260/280 nm in a UV spectrophotometer (BioPhotometer plus, Eppendorf). Genomic DNA was removed by DNAse I treatment (RQ1 Promega). 1 μg of total RNA was reverse transcribed to cDNA using M-MLV (Promega) and 25 μM Hexamers (Fermentas). Quantitative PCR was performed using the LightCycler 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics) and the LightCycler® 480 System (Roche). In order to detect GHSR-1a expression specifically, forward 5′-TGCTGGCTGTAGTGGTGTTTGC-3′ and reverse 5′-AGGACAAAGGACACGAGGTTGC- 3′ primers were designed using the QuantPrime web application. TBP expression was used as the housekeeping gene. The data represent the results of RNA analyses from 3 different independent experiments. The data depicted represents the relative mRNA levels calculated with a $2-\Delta\Delta Ct$ method where ΔCt = Ct gene of interest−Ct TBP.

2.6. Statistical analysis

Results are expressed as means ± (SD). Groups were compared using the Student's t-test analysis. A p-value <0.05 was considered statistically significant, and values marked with *. ** correspond to a p-value <0.01.

3. Results

Saos-2 cells are an osteosarcoma differentiated cell line, while MG63 cells are an osteosarcoma preosteoblastic cell line that can be pushed into a more mature state by culturing for 3 weeks in an osteogenic medium (OM). Therefore, Saos2 and MG63 cell lines allow testing of the effects of AG depending on osteoblastic differentiation states.

3.1. AG stimulates MG63 mineralization only after MG63 differentiation

MG63 and dMG63 cells were cultured for 21 days with or without Acylated Ghrelin (AG), or with OM alone as a control. As shown in Fig. 1A with alizarin red staining to visualize calcium deposition, OM

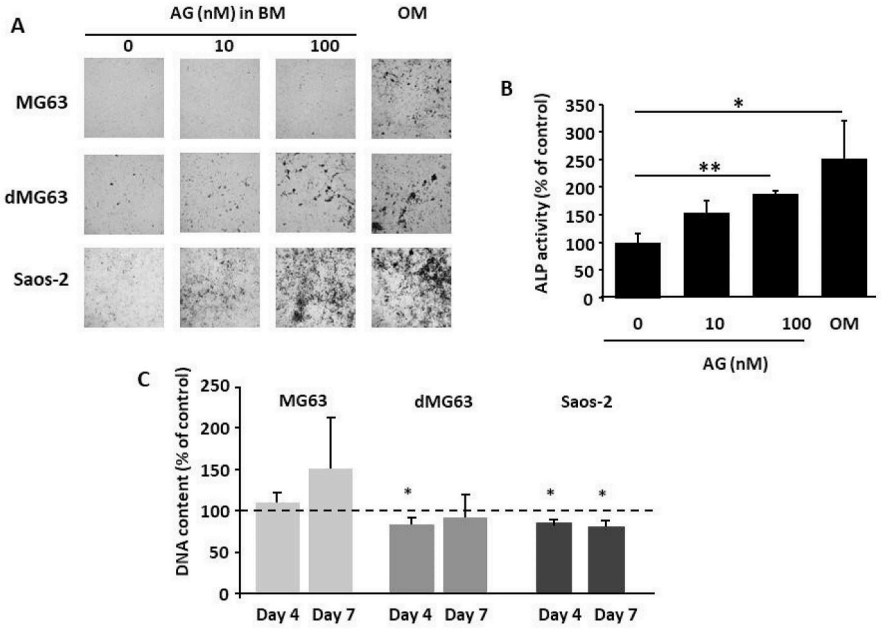

Fig. 1. Effect of AG depends on the state of osteoblastic differentiation.
MG63, dMG63 or Saos-2 cells were cultured in BM, BM with AG (10 or 100 nM), or OM. (A). Microscopic observations of alizarin red staining after 21 days of culture. (B). Alkaline phosphatase (ALP) activity (normalized with the DNA content) of Saos-2 cells tested after 7 days of culture. (C). DNA content of MG63, dMG63, and Saos2 cells cultured in BM for 4 and 7 days. The data presented is expressed as percentages of the mean value obtained from cells cultured in BM alone. Data is expressed as the mean ± SD from 3 independent experiments.

was able to similarly induce mineralization of MG63 and dMG63 cells. However, depending on dose, AG stimulated the formation of calcified nodules in dMG63 cells, but not in MG63 cells. Likewise, DNA content increased upon AG exposure after 4 and 7 days of culture in MG63 cells but decreased in dMG63 cells (Fig. 1C). Therefore, AG seems to stimulate the proliferation of preosteoblastic cells and the mineralization of more differentiated cells.

3.2. AG stimulates mineralization of mature Saos-2 osteoblastic cells

Saos-2 cells were cultured for 21 days in basic medium, with or without different AG concentrations, or in an osteogenic medium (OM). As observed with the Alizarin red staining (Fig. 1A, bottom line), AG, depending on dose, stimulated the formation of calcified nodules by Saos-2 cells, similar to the OM. ALP activity of Saos-2 cells was also tested after 7 days of culture with the medium and AG renewed every 2 days. AG demonstrated a significant dose-dependent stimulatory effect on ALP activity in Saos-2 cells (Fig. 1B). AG decreased the DNA content of Saos-2 cells after 4 or 7 days of culture (Fig. 2C). As observed in dMG63, the effect of AG on mature osteoblastic Saos-2 cells appeared to be double: stimulating the differentiation of Saos-2 cells and slightly decreasing their proliferation.

3.3. The GHS-R/GHS-R1a receptor is highly expressed in mature osteoblastic cell lines

As shown in Fig. 2A, GHS-R protein expression, analyzed by Western blot, was higher in mature dMG63 and Saos-2 cells than in undifferentiated MG63 cells. Moreover, GHS-R protein expression increased along with the osteoblastic differentiation of MG63 cells towards dMG63 in

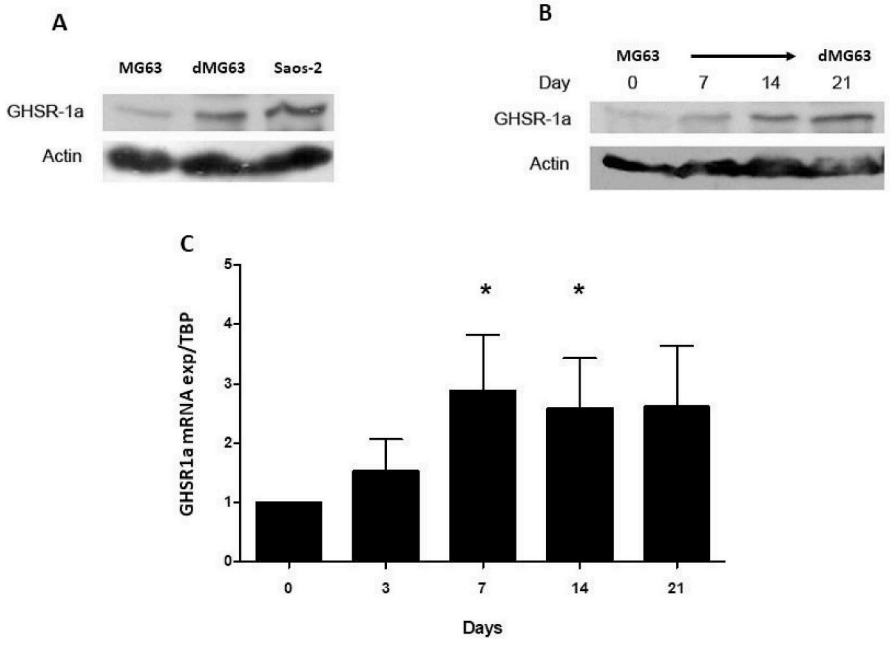

Fig. 2. GHS-R/GHS-R1a expression increase during differentiation.
Immunoblot with specific anti-GHS-R and anti-actin antibodies of Saos-2 and MG63 cells grown in BM, MG63d were grown in OM for 21 days (A and B). mRNA expression of the GHS-R1a receptor in MG63 grown in OM for 0, 7, 14 or 21 days measured by RT-PC, percentages of the mean value obtained from cells on day 0 (C). Data is expressed as the mean ± SD from 3 independent experiments.

cells cultured up to 21 days in OM (Fig. 2B). Reverse transcription analysis confirmed specific increase expression of GHS-R1a mRNA in MG63 cells during differentiation. Significant increase in expression started after 7 days of culture in OM, with a 2.8-fold increase and persisted with a 2.6-fold increase after 21 days (Fig. 2C).

3.4. AG signals through the Gi pathway in mature Saos-2 cells via GHS-R

To understand the intracellular pathways elicited, we investigated AG effects on the cAMP content of Saos-2 cells. The cAMP content of Saos-2 cells decreased significantly in a dose-dependent manner after 0 min exposure to AG (Fig. 3A). Similarly, the cAMP content of Saos-2 cells, previously stimulated by forskolin, was drastically decreased in a dose dependent manner (Fig. 4). Preincubation of the Saos-2 cells overnight with pertussis toxin (PTX), a known Gi inhibitor, prior to AG stimulation, canceled the inhibitory effect of AG on forskolin-induced cAMP production (Fig. 3B). Similarly, this effect was abolished in Saos-2 treated with GHRP6 [D-Lys], a specific inhibitor of GHS-R, prior to AG stimulation (Fig. 3C). Altogether, these results suggest that, in mature osteoblastic Saos-2 cell lines, AG, through GHS-R, activates Gi pathways and inhibits cAMP production by adenylate cyclase.

3.5. AG signals via GHS-R/Gi pathway in differentiated MG63 cells only

In pre-osteoblastic MG63 cells, AG was unable to decrease the level of forskolin-stimulated cAMP. We even observed a slight but significant increase, indicating that AG may induce cAMP production. In addition, no influence of GHRP6 [D-Lys] was observed, indicating virtually no GHS-R/Gi signalization pathway at this stage of differentiation (Fig. 3D). After 21 days of differentiation in OM, dMG63 demonstrated an ability to lower forskolin-induced cAMP production after AG exposure. Like in Saos-2, AG lost the inhibitory effect on cAMP after the previous blockade by GHRP6 [D-Lys] (Fig. 3D). Altogether, these results suggest that AG, through GHS-R, activates Gi proteins to inhibit cAMP production by adenylate cyclase only in mature osteoblastic cells.

3.6. Resistance to AG/Gi/cAMP pathway is observed in osteoblastic AIS cells

We finally investigated the effect of AG on primary human osteoblastic cells of patients with AIS and controls. After 10 min AG exposure,

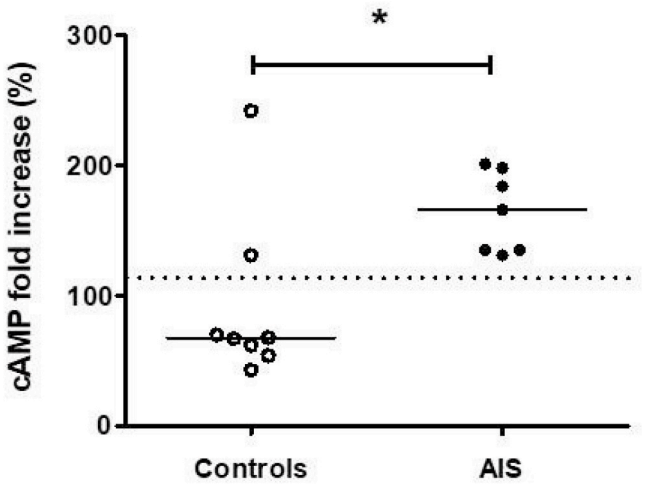

Fig. 4. AG signals through the Gi pathway in a mature osteoblastic cell line via GHS-R.
AIS and control cells were incubated with forskolin (10^{-5} M), AG (10 nM) was added to the medium for 25 min, and intracellular cAMP concentrations were measured. Expression as percentages of fold change with forskolin alone.

AG significantly decreased forskolin-induced cAMP production of control cells (Fig. 4), but was unable to decrease forskolin-induced cAMP production in AIS osteoblastic cells. These results suggest resistance to AG pathway in AIS cells.

4. Discussion

This work suggests strongly that the effect of AG is dependent on the state of differentiation of the osteoblastic cells. While AG slightly stimulated the proliferation of preosteoblastic MG63 cells, it stimulated the mineralization of MG63 previously differentiated 3 weeks in OM. Furthermore, AG induced mineralization of more differentiated cell, Saos-2, but not proliferation. AG has been suggested to directly influence *in vitro* osteogenesis by triggering osteoblast proliferation and differentiation [11–15,33]. In vivo and *in vitro* experiments have suggested that osteoblast response to AG depends on their developmental stage [16,34]. Delhanty et al. data showed that the proliferative response to

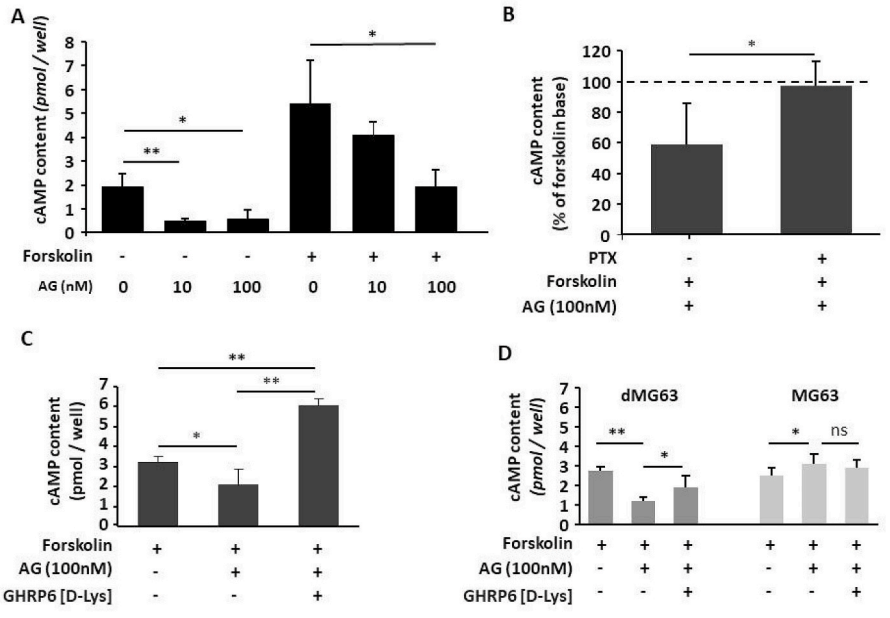

Fig. 3. AG signals through the Gi pathway in a mature osteoblastic cell line via GHS-R.
Intracellular cAMP measurement in different conditions. (A) Saos-2 were incubated with the vehicle (DMSO at 0.1% v/v) or with forskolin (10^{-5} M), and AG (0, 10 or 100 nM) was added to the medium for 10 min. (B). Saos-2 cells were incubated with or without pertussis toxin (PTX) at 1 μM overnight, then stimulated with forskolin (10^{-5} M) and 100 nM AG. Expression as percentages of the cells without AG. (C) Saos-2 cells were incubated with the GHSR inhibitor (GHRP6 [D-Lys]) at 1 μM for 2 h before incubation with forskolin (10^{-5} M) and 100 nM AG. (D). MG63 or dMG63 cells were incubated with forskolin (10^{-5} M), with or without AG, and (GHRP6 [D-Lys] was added at 1 μM 2 h before stimulation. Data is expressed as the mean ± SD from 3 to 4 independent experiments.

ghrelin declines with their progressive state of differentiation [11].

In this work, ghrelin seems to evoke Gs activation for proliferative effects and Gi activation for differentiation, but the mitotic effect of Gi coupling in osteoblasts and bone formation remains enigmatic [28]. However, ghrelin has been shown to be mitogenic with various intracellular pathways using the GHS-R1a receptor: ERK and PI3-kinase [11,14] or NO/cGMP pathway [35]. In our study, AG slightly increased the cAMP content of undifferentiated MG63 cells, suggesting that ghrelin may, like PTH/PTHR1, use cAMP production to induce mitogenesis in preosteoblasts. As this effect was not clearly blocked by the GHRP6 [D-Lys] inhibitor, our results can also suggest that a novel, unidentified acyl-ghrelin receptor that uses the Gs pathway may be present in the preosteoblastic state. This unknown receptor has been hypothesized in other cell types [1,2,5,12]. These observations could be linked to the sustained effect of Gs activity in osteoblastic cells, that produces immature bone in various disorders such as fibrous dysplasia/Mc Cune Albright syndrome [36]. Interestingly, the expression of GHS-R1a at the mRNA and protein level increased in dMG63 and were high in mature Saos-2 cells. These observations are in favor of a progressive action of GHS-R1a on differentiation. But, as we used a non-specific GHS-R antibody, we cannot completely exclude co-expression of other receptors such as the truncated receptor 1 b at the protein level. Indeed, specific expression of GHS-R1a in primary human osteoblasts has been controversial and difficult to explore [12]. However, the presence of GHS-R mRNA that recognizes both 1a and 1 b receptors has always been identified in human cell lines such as MG63 or Saos-2.

Another important result of our work shows that AG clearly decreases cAMP only in differentiated osteoblastic cells.

Moreover, the cAMP-lowering effect of AG in forskolin-stimulated Saos-2 cells was blocked by GHRP6 [D-Lys] and PTX, suggesting the involvement of GHS-R and Gi, respectively. Because of the lack of specificity of the GHRP6 [DLys] inhibitor against GHS-R1a, we cannot absolutely affirm the involvement of acylated ghrelin receptor GHS-R1a. However, expression at the mRNA level only in differentiated cells was highly specific. In parallel, only differentiated dMG63 cells show an AG-stimulated Gi pathway. We therefore suggest that GHS-R1a, the prominent putative AG receptor, increases its expression during osteoblastic differentiation and progressively uncovers a Gi pathway, which may contribute to differentiating these cells towards mineralization. The Gαi2-signaling pathway was documented in islet-β-cells, adipocytes, or smooth muscle cells [2]. A Gs pathway has also been described in mice calvaria osteoblasts [16]. GHS-R1a induces Gi signaling directly or by heterodimerization with dopamine or somatostatin receptors [2,37]. Thus, hypothetically, GHS-R1a could heterodimerize in differentiated osteoblasts, to couple with other Gi-receptor pathways elicited in osteoblasts by melatonin, lysophosphatidic acid, sphingosine-1-phosphate, cannabinoid, or apelin [28]. Moreover, oligomeric complexes between GHS-R1a and –R1b may promote changes in ghrelin-induced signaling between $G_{i/o}$ and $G_{s/olf}$, as described [38]. Indeed, all of these hypotheses remain to be explored. GHRP6 [D-Lys] appears to potentiate cAMP production in Saos cells, but not in MG63 cells possibly because of its constitutive activity that could be only present in Saos cells. It would be interesting to test other antagonists with an appropriate inverse agonist effect.

Finally, we found that primary osteoblastic cells from AIS elicited a resistance to the cAMP-lowering effect of forskolin induced AG compared to osteoblast controls. A microarray comparing the two osteoblastic populations showed no difference in the expression of GHSR (data not shown). A similar observation was made with melatonin signalization [39], which suggests a more general Gi resistance in AIS osteoblasts. We have previously reported a two-fold increase of ghrelin in the serum of AIS patients, suggesting a receptor resistance to ghrelin [8] that could correlate to the progression of scoliosis [32]. In line with our results, the mechanisms involved could be a lack of differentiation and/or a lack of a functional GHS-R1a/Gi pathway in osteoblasts from AIS which could explain the osteopenic phenotype observed in 30% of AIS patients. These results suggest again a need for precise cross-talk between Gs and Gi pathways for optimal control of osteogenesis [26–28,31]. The possible link between *in vitro* findings and a pathology needs more investigation to fully define the role of this signaling pathway in AIS.

In conclusion, these results suggest that AG stimulates differentiation of mature osteoblasts via the GHS-R1a activation with a Gi-cAMP-lowering effect. AG can contribute to osteoblast proliferation in undifferentiated cells by increasing the cAMP tone. We suggest that this dual intracellular effect may have a significant role within the concert of ligands eliciting the Gi/Gs pathway to allow the full process of maturation. Thus, the dysregulation of this signal observed in AIS osteoblastic cells may explain part of the disease.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100782.

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