Revista de Osteoporosis y Metabolismo Mineral

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10.20960/RevOsteoporosMetabMiner.00122

05/22/2026

OR 00122

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Fallo del estímulo mecánico como efecto intrínseco celular de líneas celulares osteoblásticas con mutaciones Gba1 utilizadas como modelos de la enfermedad de Gaucher ósea

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Acknowledgments: We are thankful to Carmine Settembre for hosting NMG in his lab and helping her in the KO line's generation.

CRediT authorship contribution statement: J. D. P., S. B., N. M. G., N. G. G., D. G., and R. R. were involved in the study's conceptualization and design. J. D. P., L. C., M. C., and E. M. G. performed the main assays. J. A. A., A. R. G., S. H. J., and E. M. G. did the fluid-flow assays. X. N., and D. O. contributed to project administration and funding acquisition. S. B., D. G., R. R., and N. G. G. were involved in validation and supervision. The first draft of the manuscript was written by J. D. P., S. B., N. G. G., R. R. and all authors contributed to, read and approved the final version.

Funding: This research was supported by grants FEIOMM 2019, Grants PID2019-107188RB-C21, PID2022-141461OB-I00 funded by MICIU/AEI/ 10.13039/501100011033 and "ERDF/EU". This project was supported by CIBERER (U720) and CIBERFES [CB16/10/00245] from Instituto de Salud Carlos III, and by SGR2021/1093 and SGR2021/00043 from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR). NMG was recipient of a FI (2017FI_B 01107) and JDP was recipient of FI-SDU (2020FISDU 00517) predoctoral fellowships from AGAUR. JDP obtained travel grants from the Montcelimar Foundation and the GEMSTONE-COST Action (CA18139).

Data availability: Data is contained within the article or supplementary material. Other raw data are available from the corresponding author upon request.

Supplementary data: Supplementary data to this article can be found online at https://doi.org/xxxxx

Conflicts of interest: The authors declare no conflict of interest.

Artificial intelligence: The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies were used in the writing or preparation of this manuscript.

Received: 24/04/2026
Accepted: 18/05/2026

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ABSTRACT

Background: Gaucher disease (GD) is caused by mutations in the *GBA1* gene generating lysosomal accumulation of glucosylceramides. Bone phenotypes related to GD include Erlenmeyer flask deformity, osteopenia, osteosclerosis, osteonecrosis, and bone pain. Although the osteoblastic function has been previously evaluated in GD, there are no studies assessing the osteoblast function under mechanical stimulus.

Objectives: we aimed to *in vitro* generate an osteoblastic cellular model of GD and to evaluate the effect of the mechanical stimulus in the performance of this model.

Material and methods: *GBA1* was knocked-out with CRISPR/Cas9 and two ROS17/2.8 clones displaying no GBA1 enzymatic activity were selected. ROS wt and ROS-Gba1KO cells were then plated and submitted to mechanical stress by laminar fluid flow with a shear stress of 10 dynes/cm^2, 8 Hz, for 10 minutes in a Flexcell® Streamer® Shear Stress Device. The other half were kept in static conditions. Finally, cells were cultured with fresh medium for an additional 18 h to perform RNA extraction and qPCR of osteoblastic biomarkers. During the process, cell viability and proliferation were observed without significant changes between the different experimental conditions.

Results: as is expected, mechanical stimulus induced osteoblast differentiation in all tested cell lines, observed by the increased mRNA levels of *Runx2* and *Bglap* (osteocalcin). However, expression of *Opg* (osteoprotegerin) was significantly reduced in KO cells compared to control.

Conclusion: in conclusion, lack of *GBA1* activity reduces osteoprotegerin expression in mechanically stimulated osteoblast models, suggesting an alteration of the osteoblast-osteoclast cross-talk and a subsequently impaired bone remodeling in GD.

Keywords: Gaucher disease. *GBA1* mutations. Osteoblasts.

RESUMEN

Introducción: La enfermedad de Gaucher (EG) está causada por mutaciones en el gen *GBA1* que generan acumulación lisosomal de glucosilceramidas. Los fenotipos óseos relacionados con la EG incluyen deformidad en matraz Erlenmeyer, osteopenia, osteoesclerosis,

osteonecrosis y dolor óseo. Si bien la función osteoblástica se ha evaluado previamente en la EG, no existen estudios que evalúen la función osteoblástica bajo estímulo mecánico.

Objetivos: Nuestro objetivo fue generar un modelo celular osteoblástico *in vitro* de la EG y evaluar el efecto del estímulo mecánico en la función de este modelo.

Material y métodos: Se suprimió la actividad de *GBA1* mediante CRISPR/Cas9 y se seleccionaron dos clones ROS17/2.8 sin actividad enzimática de GBA1. Las células ROS wt y ROS-Gba1KO se sembraron y sometieron a estrés mecánico mediante flujo laminar con una tensión de cizallamiento de 10 dinas/cm², 8 Hz, durante 10 minutos en un dispositivo de tensión de cizallamiento Flexcell® Streamer®. La otra mitad se mantuvo en condiciones estáticas. Finalmente, las células se cultivaron con medio fresco durante 18 h adicionales para realizar la extracción de ARN y la qPCR de biomarcadores osteoblásticos. Durante el proceso, se observó viabilidad y proliferación celular sin cambios significativos entre las diferentes condiciones experimentales.

Resultados: como era de esperar, el estímulo mecánico indujo la diferenciación de osteoblastos en todas las líneas celulares analizadas, como se observó en el aumento de los niveles de ARNm de *Runx2* y *Bglap* (osteocalcina). Sin embargo, la expresión de *Opg* (osteoprotegerina) se redujo significativamente en las células KO en comparación con el grupo control.

Conclusión: la falta de actividad de *GBA1* reduce la expresión de osteoprotegerina en modelos de osteoblastos estimulados mecánicamente, lo que sugiere una alteración de la comunicación osteoblasto-osteoclasto y, por tanto, una remodelación ósea deteriorada en la EG.

Palabras clave: Enfermedad de Gaucher. Gen *GBA1*. Osteoblastos.

INTRODUCTION

Gaucher Disease (GD, OMIM #230800, ORPHA355) is a rare autosomal recessive genetic disease caused by biallelic pathogenic variants in the *GBA1* gene (GRCh37/hg19 Chromosome 1: 155,204,239 to 155,214,653) , producing a defective activity of glucocerebrosidase (GCase; EC 3.2.1.45; *GBA1*) and generating an accumulation of

glucosylceramide (GlcCer) and its deacylated metabolite glucosylsphingosine (GlcSph), in the lysosomes of the monocyte/macrophage system (1).

The characteristic manifestations of the disease, including splenomegaly, hepatomegaly, cytopenia, and bone lesions, are due to the infiltration of Gaucher cells in the spleen, liver, and bone marrow, respectively (2). Gaucher cells are giant macrophages containing enlarged lysosomes due to the accumulation of glucosylceramide, the undegraded substrate. These cells cause damage to the tissues in which they are found and exhibit increased activity of various lysosomal acid hydrolases, including acid phosphatase (TRAP), angiotensin-converting enzyme (ACE) and lysozyme. Furthermore, one of the most characteristic features of Gaucher cells is their ability to release the enzyme chitostrioseidase and numerous cytokines (3). GlcCer can be metabolized by acid ceramidase into glucosylsphingosine (GlcSph), which is a typical consequence of GCase deficiency. GlcSph is metabolized into sphingosine and high levels of this metabolite are toxic to bone and may cause neuronal dysfunction and death (4). Gaucher disease phenotype is variable, but three clinical forms have been identified according to the severity of neuronopathic involvement, named non-neuronopathic (GD1, OMIM 230800); acute neuronopathic (GD2, OMIM 230900); and chronic or subacute neuronopathic (GD3, OMIM 231000) (5-7).

Type I Gaucher disease (GD1) presents a wide spectrum of clinical severity, ranging from affected infants to asymptomatic adults. GD1 also presents musculoskeletal conditions, these include bone marrow infiltration by Gaucher cells, bone infarction, recurrent avascular osteonecrosis, microvascular occlusion in the bone, cortical thinning, impaired bone remodeling, osteolytic lesions and bone density loss. Some mechanisms affect the bones, such as bone pain, increased fracture susceptibility, joint damage with secondary osteoarthritis, bone deformity and disability, affecting the quality of life of the patients and sometimes necessitating orthopedic surgeries. It has been demonstrated that up to 90 % of Gaucher disease patients have bone involvement, as evidenced by imaging studies. The most significant finding is the appearance of the Erlenmeyer flask deformity, with the femur being one of the most affected bones (8,9).

GD1 is the most frequent type of the disease and presents skeletal symptoms in up to 80 % of patients, which significantly reduces their quality of life. Bone involvement may be limited to asymptomatic failure to remodel (Erlenmeyer flask deformity). Bone remodeling involves

the crosstalk between bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) and is essential for proper bone homeostasis. Mechanical stimulus plays an important role through the activation of osteoblasts to produce osteoprotegerin (*Opg*), which protects against osteoclast-mediated bone resorption (10,11). Bone involvement in GD may also include medullary and corticocancellous infarcts, pathological fractures, osteonecrosis of humeral or femoral heads, and collapse of vertebrae (12-14). Nowadays, the precise mechanisms underlying the bone physiopathology in GD are still unclear.

Lysosomes, as part of the autophagy pathway, play important roles in regulating osteoblast differentiation and osteoclast maturation (15). On the one hand, autophagy protects osteoblasts from apoptosis and may be critical for osteogenic differentiation and for bone formation.

In this study, we aim to produce osteoblast-like cells with loss-of-function mutations in the *Gba1* gene using CRISPR/Cas9, and to evaluate their intrinsic performances in order to broaden our understanding of the bone phenotype in GD.

MATERIALS AND METHODS

Generation of Gba1KO models using CRISPR/Cas9

The rat osteosarcoma cell line ROS 17/2.8; RRID:CVCL_0508 was used as a model of osteoblast-like cells in its wild-type and *Gba1* knockout (ROS-*Gba1*KO) versions. *Gba1* editing was performed at Organelle Homeostasis Laboratory of TIGEM (Naples, Italy) using the pSpCas9(BB)-2A-GFP (PX458) plasmid and Sigma-Aldrich Synthetic guide RNA (RN00064-0684536) (sgRNA) (Suppl. Table I) which targets exon 3 of the *Gba1* gene. For guide selection, off-target site prediction was carried out using Benchling (https://www.benchling.com/) (Suppl. Table II). The sgRNA was cloned into the pSpCas9(BB)-2A-GFP vector following the protocol of Ran et al. 2013 (16). After the sgRNA was cloned and tested, the cells were seeded one day before transfection in a 6-well plate ($3,5 \times 10^5$ cell/well). Reverse transfection was performed using 2500 ng of pSpCas9 plasmid containing sgRNA, 7.5 µl of Lipofectamine™ 3000 and 5 µl of Lipofectamine™ P3000 reagent (Invitrogen-Thermo Fisher Scientific, USA) according to the manufacturer's protocols. After 48-72 h the edited pool was sorted by single-cell sorting in flow cytometry (BD Biosciences LSR II flow cytometer, San Jose, CA, USA). From a single clone, cells were cultured to sufficient numbers to perform DNA extraction to identify edited clones by Sanger

sequencing. Edited clones predicted to lead to KO proteins were analyzed by western blot (WB) in order to confirm lack of GCase expression and GCase activity was measured for selected clones.

Cell cultures

ROS wt and ROS-*Gba1*KO cells were cultured and maintained in DMEM F-12 medium (Gibco) containing 10 % fetal bovine serum (Gibco, Origin: Brazil), and 1 % penicillin/streptomycin (Gibco) (complete media), in a humidified atmosphere containing 5 % CO_2 at 37 °C. Osteoblast viability was assessed using the MTT assay (Sigma). Briefly, 80 000 cells were cultured in 24-well for 3 days and the MTT reagent 0,45 mg/ml was added. Absorbance was read at 560 nm after incubation at 37 °C for 4 hours. Three independent replicates were performed.

Western blot

In order to evaluate expression levels of GCase, cells were washed once with PBS and lysed in RIPA buffer using cell scrapers. The protein extracts were quantified using the PierceTM BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Proteins (20 µg/lane) were separated by electrophoresis in an SDS-polyacrylamide gel (SDS-PAGE) and transferred to a hydrophobic PVDF transfer membrane (Merck-Millipore, Burlington, MA, USA). We used the appropriate primary antibody against GCase/GBA 2 µg/mL (GBA 2E2 WH0002629M1, Sigma-Aldrich, St. Louis, MO, USA), and the antibody against actin 1:200 (A2066, Sigma-Aldrich, St. Louis, MO, USA) was used as the loading control. The membranes were developed using a peroxidase-conjugated secondary antibody anti-rabbit IgG (A0545) 1:10 000 for GCase/GBA, and actin antibodies. Each one of these quantifications was used to perform an independent experiment, producing a total of 3 experiments for each biological replica.

Glucocerebrosidase enzymatic activity

Glucocerebrosidase (GCase) enzymatic activity was measured using the fluorogenic substrate 4-methylumbelliferyl--d-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA). The total amount of protein in cell lysates was determined using the Bradford assay (BioRad, Hercules, CA, USA), following manufacturer's instructions. In each well of a 96 well plate,

24 µL containing 24 µg of protein was incubated at 37 °C for 30 minutes. After that, 100 µL of McIlvaine's buffer (Citrate-Na_2HPO_4 solution 150 mM; Taurocholate 0,2 %; Triton X-100 0,1 %; BSA 0,1 %; substrate 1,25 mg/ml; pH 5,2) were added and the plate was incubated again at 37 °C for 30 minutes. The reaction was stopped with NaOH-glycine buffer 0.3 M pH 10.4 and the fluorescent product was quantified using a fluorimeter (Thermo Scientific Fluoroskan Ascent FL Microplate Fluorimeter and Luminometer) at an excitation wavelength of 365 nm and emission of 495 nm. Quantification was performed in comparison to a reading without adding substrate to the cells (blank). Readings lower than the blank are shown as 0. This assay was performed once for each selected clone.

Fluid-flow assay

ROS wt and ROS-*Gba1*KO cells were plated at 2×10^4 cells/cm^2 on glass slides pre-treated with collagen (FlexCell, Hillsborough, NC) and cultured with DMEM-F12 complete medium. After 24 h of incubation half of the cells were submitted to mechanical stress by laminar fluid flow (FF) with a shear stress of 10 dynes/cm^2, 8 Hz, for 10 minutes in a Flexcell® Streamer® Shear Stress Device. The other half were kept at static conditions (SC). Finally, cells were cultured with fresh medium for an additional 18 h to perform RNA extraction and qPCR (Suppl. Table I). During the process, cell viability and proliferation were observed without significant changes between the different experimental conditions. In brief, non-adherent cells were collected and pooled with trypsinized adherent cells and counted in a Neubauer chamber. The number of living cells and the cell viability were determined by trypan blue exclusion assay, in which refringent cells were considered living cells whereas cells exhibiting intracellular blue staining were considered dead. Three independent replicates were carried out.

RT-PCR

Total RNA was extracted from ROS (wt and KOs) cells submitted to FF or SC with High Pure RNA Isolation Kit (Roche, Basel, Switzerland) following the manufacturer's recommendations. 2 µg of RNA were used for retro-transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), followed by RT-qPCR with LightCycler® 480 Probes Master (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland) following the commercial protocol. Primers were

designed from available mouse and rat sequences using the primer analysis software Primer3 (Suppl. Table I). A mouse and rat-*Gapdh* gene was used as an internal control. The comparative threshold (Ct) method was used for data analysis expressed as 2-ΔCt. Three independent experiments were carried out for each biomarker.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA), applying unpaired, paired *t*-tests, Mann-Whitney-U test or ANOVA as appropriate. Data are expressed as mean ± SD and are representative of three or more independent experiments.

RESULTS

Generation of *Gba1* gene knockout in osteoblast-like cell lines

We generated and characterized *Gba1* knockout rat osteosarcoma cell lines (ROS-*Gba1*KO) as putative osteoblastic cell models of GD. We obtained two KO clones from ROS cells. The first one (ROS-*Gba1*KO1) carried an homozygous frameshift deletion of two nucleotides (NM_001127639.1: c.256_257del; p.Leu86AsnfsTer44) and the second one (ROS-*Gba1*KO2) carried an homozygous frameshift deletion of one nucleotide (NM_001127639.1: c.258del; p.Leu87ProfsTer11) (Fig. 1A). These deletions were validated by Sanger sequencing (Suppl. Fig. 1).

The ROS-*Gba1*KO cells showed no GCase protein expression, as determined by western blot (Fig. 1B), and displayed less than 1 % residual GCase activity (Fig. 1C).

Functional characterization of the generated KO osteoblasts under mechanical stimulus

First, we checked cell viability and proliferation without observing any significant difference between the KO clones and the wild type cell line (Fig. 2).

Given the well-established anabolic effects of mechanical stimuli on bone, we analyzed the response of KO cells to mechanical stimulation in comparison with wt cells. Mechanical stimulation induced a significant increase in *Runx2* and *Bglap* mRNA expression across all cell types, with no significant differences observed between KO and wt cells. However, for *Runx2*, a statistically significant difference was detected between wt cells and one KO line (ROS-Gba1KO2) under static conditions. In contrast, *Opg* expression exhibited a distinct

pattern: wt cells showed a threefold increase following mechanical stimulation, whereas this stimulatory effect was significantly attenuated or absent in KO cells (Fig. 2).

DISCUSSION

Here we aimed to generate and characterize an osteoblastic cell model of GD to understand the mechanisms underlying the skeletal symptomatology associated with this pathology. We used CRISPR/Cas9 gene editing to generate *Gba1* knockouts in rodent bone cell lines since they do not harbor the *GBA* pseudogene. After validating the models, we evaluated the effect of a mechanical stimulus in these Gba1 knockout cells by determining the expression of osteoblastic biomarkers.

Regarding the osteoblastic function of ROS-*Gba1*KO cells, we analyzed the expression of *Runx2*, *Bglap*, and *Opg*, which are involved specifically in the differentiation and function of osteoblasts. In our mechanical loading tests, no consistent differences were observed in the expression of either *Runx2* or *Bglap,* compared to the wt cells, both before and after the mechanical stimulus. These bone markers encompass three crucial pathways of osteoblasts: *Runx2* is a transcription factor that plays an essential role in both osteoblast differentiation and expression of osteoblast-specific genes. *Runx2* is crucial for the commitment of mesenchymal stem cells to the osteoblast lineage and positively influences early stages of osteoblast differentiation. Hence, it is an early marker of osteoblast differentiation. Osteocalcin (*Bglap*) is a hormone secreted solely by osteoblasts and is thought to play a role in the body's metabolic regulation. It is expressed by the mature osteoblast and therefore, it is a marker of a later differentiation stage. Finally, Osteoprotegerin (*Opg*) is also secreted uniquely by osteoblasts and binds specifically to *Rankl* as a decoy receptor. The binding of *Rankl* with its receptor *Rank*, in the membrane of osteoclast precursors, is essential for osteoclast formation and activation. Osteoprotegerin regulates bone remodeling by decreasing osteoclast formation and thus, it is used as a marker of osteoblast function (10,17-19).

The *in vitro* studies performed to analyze the status of osteoblasts in GD have reported controversial results. Some of them, including studies with iPSC-derived osteoblasts, osteoblast-like cell lines treated with an inhibitor of glucocerebrosidase (conduritol-B-epoxide, CBE) or cells obtained directly from patients with GD, have observed altered osteoblast function, with significantly decreased biomarkers like *Runx2*, *Alp*, *Col1*, *Bglap* (20-

22). On the other hand, Lecourt et al. (23) in an *in vitro* model with CBE-treated and untreated mesenchymal stem cells (MSCs), observed impaired proliferation in treated compared to untreated MSCs, but when they analyzed osteoblast differentiation by the level of *Runx2* and Osterix mRNA expression, they found no significant differences between treated and untreated cells after 7 days of culture in osteoblastic medium. In the same way, studies of a cohort of untreated type 1 GD patient samples showed no statistically significant differences in bone biomarkers such as osteocalcin and bone ALP isoenzyme (24,25), or osteocalcin, bone-specific alkaline phosphatase, N-telopeptide cross-links and deoxypyridinoline (D-PYD) (26), which were within the normal range compared with the respective controls. Our results of osteoblast mRNA biomarkers, with no differences between disease and control, align with them.

Regarding proliferation defects in osteoblasts, several studies have repeatedly observed less proliferation both in animal and cell models of Gaucher (21-23,27). Given the essential role of the Wnt pathway in osteoblastogenesis (28) it is not surprising that defects in this pathway have been described in some of the models (21,23,29) which could explain the proliferation defects. In the present work, we have not observed evident proliferation alterations in our ROS KO lines, probably due to their osteosarcoma origin, their immortalized nature, and the culture conditions *in vitro*.

Our Gaucher bone cell models have several limitations, primarily related to their origin as transformed cell lines and their maintenance as isolated monocultures. These features preclude the assessment of cell-cell interactions and fail to fully recapitulate the physiological processes observed in animal models, which may limit their suitability as comprehensive models of Gaucher disease. Nevertheless, these systems are easy to maintain and, importantly, enable the investigation of intrinsic, cell type-specific mechanisms contributing to the bone phenotype. Future studies combining these cell lines in co-culture systems to better mimic the bone microenvironment may provide a more accurate characterization at the cellular level and facilitate drug testing.

As a main result, Gaucher model osteoblasts failed to upregulate osteoprotegerin compared to wild-type osteoblasts, which significantly overexpressed this marker in response to the stimulus. To our knowledge, this is a novel finding and we hypothesize that this lack of *Opg* activation may in part explain the defective cross-talk described between Gaucher bone cells, in which a superior osteoclast differentiation capacity is driven by Gaucher osteoblasts

(30,31). The lack of *Opg* expression would not result in competitive inhibition of the *Rank-Rankl* interaction necessary for osteoclast differentiation, ultimately disrupting bone homeostasis (32).

CONCLUSION

In conclusion, in the context of our cell model, our findings point out to an impaired osteoblast-osteoclast cross-talk mediated by osteoprotegerin which could affect the bone remodeling and in part, to explain the bone phenotype observed in Gaucher disease.

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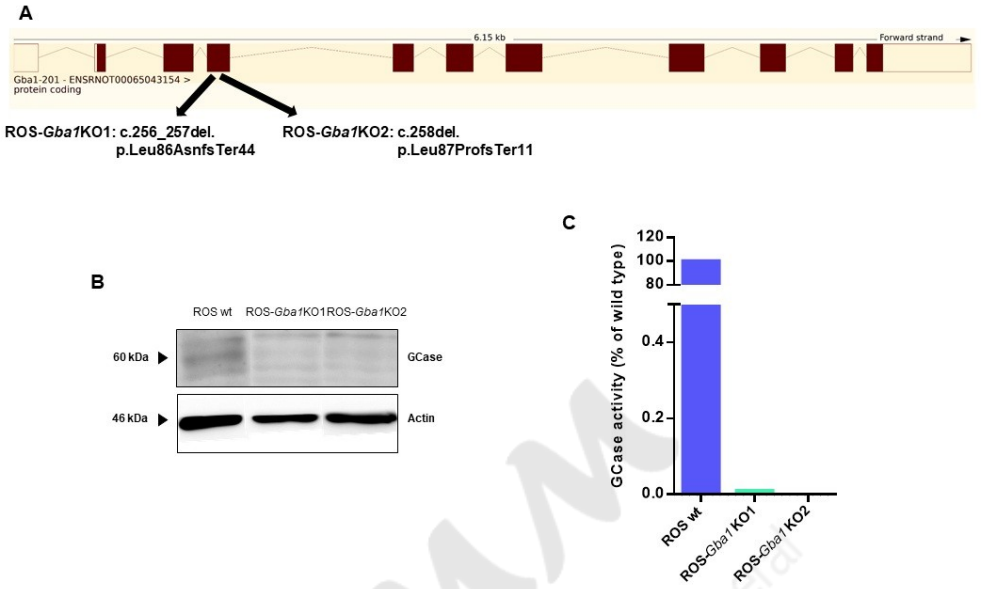

Figure 1. Generation and characterization of Gaucher osteoblastic models. (A). Schematic representation of *Gba1* gene from *Rattus norvegicus* with the localized mutations. An homozygous two-nucleotide deletion (256-257) in the ROS-*Gba1*KO1 clone and an homozygous one-nucleotide deletion (258) in the ROS-*Gba1*KO2 clone. (B). WB analysis of GCase expression in ROS cells (wt and KO). The KO clones showed lower levels of GCase expression in comparison to wt. (C). GCase enzymatic activity of ROS cells (wt and KO). The GCase enzymatic activity of selected KO clones was greatly decreased compared to wt cells. The results correspond to one biological replicate for each cell type.

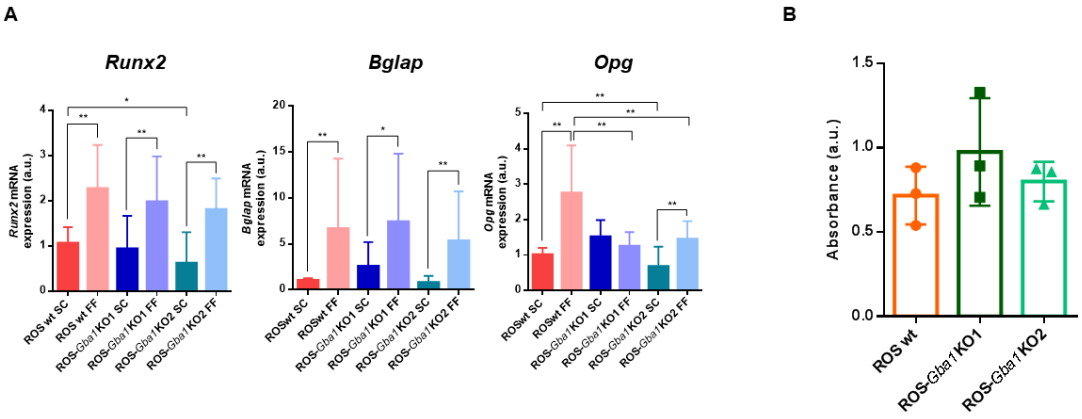

Figure 2. Mechanical stimulus assay (fluid-flow) with ROS-*Gba*KO cell models. A. Expression of osteoblastic marker mRNA after mechanical stimulus assay. Results are expressed as mean ± SD of three independent experiments. B. Cell viability. MTT results are expressed as mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.001$. SC: static control; FF: fluid-flow (mechanically stimulated sample).