The Role of GPR56 in Neurofibromatosis type 1 Secondary to Scoliosis

Siyi Cai
Peking Union Medical College Hospital
Yuemei Yang
Peking Union Medical College Hospital
Binghan Jia
Peking Union Medical College Hospital
Zhihong Wu
Peking Union Medical College Hospital
Jianguo Zhang
Peking Union Medical College Hospital
Jianxiong Shen
Peking Union Medical College Hospital
guixing qiu (doctor2018@yeah.net)
Peking Union Medical College Hospital https://orcid.org/0000-0001-7557-1252

Research

Keywords: Neurofibromatosis type 1 with scoliosis, ADGRG1/GPR56, proliferation, cell cycle, osteoblastic differentiation and osteoclast formation

Posted Date: September 14th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-74862/v1

Abstract

Background: Scoliosis is a common manifestation of neurofibromatosis type 1, causing significant morbidity. The etiology of dystrophic scoliosis in neurofibromatosis type 1(NF1) is not fully understood and its therapies are lacking. This article focused on how GPR56 affected the development of NF1 cells and the related factors of osteoblast and osteoclast.

Methods: Through RNA-sequencing, G-protein-coupled receptor (GPR56) was found highly differential expressed in the NF1 with scoliosis samples. We measured the GPR56 how affected the NF1 cells in vitro. Then we tested the influence of GPR56 in osteoclasts and osteoblasts.

Results: We reported that knockdown GPR56 promoted the proliferation and cell cycle progression of NF1 cell. Furthermore, knockdown GPR56 can inhibited the osteoblastic differentiation and osteoclast formation, but had greater influence on osteoblastic differentiation, which indicated that knockdown GPR56 can promote the deterioration of NF1 tumors, meanwhile break the dynamic balance of bone formation and bone absorption, leading to increased bone resorption and eventually scoliosis.

Conclusions: In conclusion, we found that GPR56 may inhibit the proliferation and cell cycle progression of NF1. Furthermore, GPR56 can promote osteoblastic differentiation and osteoclast formation, but had greater influence on osteoblastic differentiation, which indicated that knockdown GPR56 can promote the deterioration of NF1 tumors, meanwhile break the dynamic balance of bone formation and bone absorption, leading to increased bone resorption.

Background

Neurofibromatosis type 1(NF1) is an autosomal dominant genetic disease [1]. The current clinical diagnosis of NF1 was established in 1987 by the National Institutes of Health[2]. The incidence of NF1 is 1/4-5000, and the incidence in newborns is about 1/2500–3000[3]. NF-1 as a multisystemic disease, NF-1 may manifest as abnormalities of the nervous tissue, bones, soft tissue, and skin[4]. Scoliosis is the most common clinical features of NF-1, with an incidence ranging from 21–49%[5, 6]. It may vary in severity from mild and non-progressive to severe curvatures[7]. NF1 with scoliosis deformity has a great impact on the patients' health and quality of life[5, 8–10].Currently, the understanding of the molecular mechanism of NF1 is mainly focused on its role in cell signal transduction, such as Ras-GTP, mTOR signaling and so on[11, 12]. And previous investigators used whole-spine magnetic resonance imaging (MRI) in conjunction with plain radiographs to delineate intraspinal and extraspinal tumors and illustrate the spinal deformity in detail[13–15].Although the cellular signal transduction pathway of NF1 has been elucidated and the diagnose of spinal malformation has been relatively matured, there is a lack of multi-system and multi-angle studies on the occurrence, development and pathogenesis of NF1 with scoliosis.

Transcriptome sequencing (RNA-seq) is a method based on second-generation sequencing for the determination of genomic expression in specific time and tissue. [16]. Through this method, we found some different expression gene. ADGRG1/GPR56 was one of these different expression genes, who was

downregulated in the NF1 with scoliosis. Adhesion G-protein-coupled receptors (GPCRs), which are an atypical class of GPCRs, are characterized by an unusually long extracellular domain that is involved in cell-to-cell and cell-to-extracellular matrix interactions[17]. In the adult brain, GPR56 is highly expressed in the dentate gyrus and subventricular zone[18]. GPR56 is down-regulated in highly metastatic melanoma-derived cell lines, which consistent with our findings in NF1 with scoliosis[19]. Tseng WY et al. reported that serum soluble GPR56 (sGPR56) levels were increased in patients of rheumatoid arthritis [20]. One study also indicated that GPR56 regulated the proliferation and invasion capacity of osteosarcoma cells[21].

The expression pattern of ADGRG1/GPR56 in the nervous system, bone disease and its relationship with the occurrence and migration of cancer indicate that it may be highly related to NF1 with scoliosis in molecular function. However, the role and specific mechanism of ADGRG1/GPR56 in the development of NF1 with scoliosis remains to be further explored. This article focused on how GPR56 affected the development of NF1 cells and the related factors of osteoblast and osteoclast. We measured the GPR56 how affected the NF1 cells in vitro. Then we tested the influence of GPR56 in osteoclasts and osteoblasts.

Materials And Methods

Human blood sample

Five pairs of human blood samples were obtained from patients who underwent Surgical treatment between 2017 and 2018 and were diagnosed with Neurofibromatosis type I (NF1) with scoliosis based on a computed tomography(CT)、Magnetic Resonance Imaging(MRI)、 CT Mylography(CTM) or histopathological evaluation. The matched control blood was obtained from volunteer. The samples were snap-frozen in liquid nitrogen and stored at -80˚C until use. No local or systemic treatment was conducted on these patients prior to surgery.

The use of human blood samples followed internationally recognized guidelines, as well as local and national regulations. All research carried out on human participants followed international and national regulations. Ethics approval for this study was obtained from the Medical Ethics Committee and all participants provided written informed consent prior to enrollment.

Antibodies

Primary antibodies were obtained from the following sources: GPR56 (ab77515, 1:1000), IGFBP3 (ab77635, 1:1000), Ras(ab52939, 1:1000), Raf1(ab137435, 1:1000), Phospho Raf1(ab173539, 1:1000), ALP(ab83259, 1:1000), OCN(ab13420, 1:1000), OPN(ab8448, 1:1000), Runx2(ab192256, 1:1000), NFATc1(ab2796, 1:1000), C-Fos(ab190289, 1:1000) were obtained from Abcam Inc. MEK1/2(#4694, 1:1000), Phospho MEK1/2(#9127, 1:1000), ERK1/2(#9102, 1:1000), Phospho ERK1/2(9101, 1:1000),

anti-mouse secondary IgG anti-goat and anti-rabbit secondary IgG anti-goat antibodies were received from Cell Signaling Technology, Inc. (1:2000).

Cell culture

The rat Schwann cells(RSC96), mouse embryonic osteoblasts cells(MC3T3) and the mouse mononuclear macrophage leukemia cells(RAW264.7) were obtained from the National Infrastructure of Cell Line Resource (Cell resource center, institute of basic medicine, Chinese academy of medical sciences) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM-H, Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37˚C with 5% CO2. The medium was changed every two days.

Osteoclast differentiation and TRAP staining

Raw264.7 cells were seeded in 24-well plates at a density of 2.5 $\times 10^4$ cells/well. After (4-6)h culturing, media were added recombinant mouse RANKL (Sigma, USA) to 50 ng/mL of the concentration ; the cultures were incubated at 37℃for 7 days. Cells were fixed with 4% paraformaldehyde and stained for TRAP activity as described previously[34]; the plates were then scanned using an Inverted microscope (CKX31) (Olympus, Japan).

RSC96, MC3T3 and Osteoclast were transfected with ADGRG1-siRNA

The Rat/Mouse-siRNA pool against ADGRG1(ADGRG-R-siRNA & ADGRG-M-siRNA) was synthesized from Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China). Transfection was conducted using RiboFECT^{TM} Transfection Reagent (Ruibo, Guangzhou, China) according to the instructions. After 72h, the transfected cells were collected for experimental determination.

RNA Sequence

Total RNAs isolated from blood samples (NF-1 with scoliosis patients and healthy people) using TRIzol reagent (Invitrogen, USA). RNA integrity and concentration were determined using Nanodrop and agarose gel electrophoresis. The purified RNA samples, with RIN (RNA Integrity Number) over 8.0, determined by Agilent 2100 Bioanalyzer (Agilent, Waldbroon, Germany) were sequenced at NovoGene corporation(Beijing, China), who constructed their digital gene expression libraries and sequenced by means of Illumina HiSeq™ 2500 platform to obtain the expression libraries of 150-nt read length. Independent triplicate cultures were sampled. The clean reads were mapped against human genome hg19 with less than two-base mismatching, using Tophat (version 2.1.1b). The normalized expression values for each gene were calculated by FPKM (expected number of Fragments per Kilo base of

transcript sequence per Million base pairs sequenced). Differential expression gene was determined by Cuffdiff package (v2.2.1). The transcript levels of genes having a P-value of less than 0.05 were significantly differential between two groups. Then, we found the gene ($P \leq 0.05$, $logFC \geq 1$ or $logFC \leq -1$) to further research by the NCBI PubMed(https://www.ncbi.nlm.nih.gov/pubmed/).

Quantitative real-time PCR

According to the manufacturer's instructions, TRIzol reagent (Invitrogen, Carlsbad, California) was using for isolating the total RNA of cells. First-strand cDNA was synthesized from 1 μg of total RNA by incubation for 1 hour at 42°C with Superscript III reverse transcriptase (Invitrogen) following oligo (dT) priming. After the reverse transcription reaction, an ABI PRISM^{R}500 system (ABI, USA) was applied to measure the real-time polymerase chain reaction (RT-PCR) according to the manufacturer's instructions. The conditions for RT-PCR were as follows: denaturation at 95°C for 10 seconds and 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. A dissociation stage was added to the end of the amplification procedure. No nonspecific amplification was observed, as determined using the dissociation curve. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The data were analyzed using the comparison Ct (2-ΔΔCt) method and expressed as the fold change relative to the respective control. Each sample was analyzed in triplicate.

Western blot analysis

We collected the cells from the plates and then used lysis buffer supplemented with protease inhibitors (10 mg/mL leupeptin, 10 mg/mL pepstatin A, and 10 mg/mL aprotinin) for extracting total protein about 30 minutes. Protein samples were collected by centrifugation at 12 000g at 4°C for 15 minutes. Then the Micro Bicinchoninic acid (BCA) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was using for determining protein concentrations. In addition, 20 μg of total protein extract was then subjected to 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly vinylidene fluoride(PVDF) membranes (Millipore, USA). After being blocked with 5% BSA, the membranes were incubated with according antibodies overnight at 4°C. A horseradish peroxidase-conjugated secondary antibody was added and visualized using an enhanced chemiluminescence detection system (Millipore, Billerica, Massachusetts) as recommended by the manufacturer.

CCK8 assay

Cell proliferation was measured by CCK8 dye reduction assay. Briefly, the transfected cells were transfected (blank control group, siRNA NC group, the siRNA - 1 group, 2 group and siRNA- 3 groups) with high-DMEM containing 10% FBS culture solution, formed cell suspension, and respectively inoculated into 96- well plates, 100μl /holes, each group of five hole. Then, at 24 h, 48 h, 72 h and 96 h, samples were

token, 10μl per hole CCK8 solution was added avoid light, continuing to culture 1 h. The absorbance was measured at 450 nm using a microplate reader.

Flow cytometric analysis

After 48 h of transfection, the cells cultured in 12-well plates were collected and fixed in 70% ethanol at −20 °C overnight. The flow cytometric analysis was carried on a BD AccuriC6 flow cytometer (BD Biosciences, San Jose, CA, USA) with a Cell Cycle Analysis Kit (Nanjing kaiji biotechnology co., LTD, China). The data were processed using the FlowJo7.6 software (Treestar Incorporated, Ashland, OR, USA).

Statistical analysis

Statistic comparisons between results from multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. For experiments involving two groups, an unpaired Student's t-test was performed. A value of $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation (SD). A P-value < 0.05 was considered statistically significant.

Results

ADGRG1 and IGFBP3 differential expressed between NF-1 with scoliosis patients and healthy controls

We identified 30299 mRNAs in all assemble transcripts. Comparing between the two groups, 780 differential expressed mRNAs were identified in which 319 were upregulated and 461 were downregulated significantly (P value < 0.05) (Table 1).Then, combining NCBI literature and gene information retrieval, we found that ADGRG1 and IGFBP3 were possibly related NF1with scoliosis[17, 19, 22–28]. Our Further analysis of clinical samples showed that the expressions of ADGRG1 and IGFBP3 both in gene and protein levels were significantly decreased in NF1 with scoliosis group than that in the control group($P < 0.001$)(Fig. 1). Compared to the control group, the gene expression level of IGFBP3 was significantly higher in the scoliosis group ($P < 0.001$), while the gene expression level of ADGRG1 was lower ($P < 0.001$) (Fig. 1A). However, in protein level, the expression of IGFBP3 and GPR56 showed no statistical significance between these two groups (Fig. 1B).

Table 1
The top 15 different expression Gene by RNA-seq

ID	GeneName	log2 FoldChange	P-value	GeneType
Down-regulated				
ENSG00000197467	COL13A1	-2.30492	9.27E-12	protein_coding
ENSG00000238268	RP11-229P13.19	-2.09398	1.25E-16	lincRNA
ENSG00000133067	LGR6	-2.07873	2.18E-15	protein_coding
ENSG00000266124	MIR5587	-1.95108	1.97E-07	miRNA
ENSG00000205336	**ADGRG1**	-1.92566	1.41E-17	protein_coding
ENSG00000146674	**IGFBP3**	-1.87312	2.50E-07	protein_coding
ENSG00000273824	RP11-81H14.1	-1.86719	3.36E-07	lincRNA
ENSG00000160318	CLDND2	-1.83407	4.92E-13	protein_coding
ENSG00000171596	NMUR1	-1.82837	1.86E-15	protein_coding
ENSG00000189409	MMP23B	-1.81728	8.79E-11	protein_coding
ENSG00000175899	A2M	-1.8	1.78E-10	protein_coding
ENSG00000126838	PZP	-1.79791	2.62E-08	protein_coding
ENSG00000273179	RP11-20I20.4	-1.77667	1.98E-08	antisense
ENSG00000159674	SPON2	-1.72624	1.10E-10	protein_coding
ENSG00000173068	BNC2	-1.72523	3.16E-08	protein_coding
Up-regulated				
ENSG00000231412	CTC-490G23.2	2.067104	1.84E-08	lincRNA
ENSG00000268734	CTB-61M7.2	1.541643	2.99E-06	lincRNA
ENSG00000168528	SERINC2	1.533816	3.07E-05	protein_coding
ENSG00000186431	FCAR	1.511637	4.46E-06	protein_coding
ENSG00000162407	PLPP3	1.465099	2.63E-05	protein_coding
ENSG00000274641	HIST1H2BO	1.421581	2.22E-05	protein_coding
ENSG00000225981	AC102953.4	1.351399	0.000177	antisense
ENSG00000173585	CCR9	1.331759	0.000132	protein_coding
ENSG00000105048	TNNT1	1.331393	0.000157	protein_coding
ENSG00000168916	ZNF608	1.327796	0.000498	protein_coding

ID	GeneName	log2 FoldChange	P-value	GeneType
ENSG00000138772	ANXA3	1.327509	0.00029	protein_coding
ENSG00000130508	PXDN	1.308608	0.000512	protein_coding
ENSG00000257335	MGAM	1.304767	0.000331	protein_coding
ENSG00000112290	WASF1	1.296047	6.70E-07	protein_coding
ENSG00000112137	PHACTR1	1.294222	1.41E-05	protein_coding

Knockdown of GPR56 could promote the proliferation and cell cycle progression of RSC96 cells

In order to further investigate the role of GPR56 serves during the development of NF1 with scoliosis, the changes of cell viability and cell cycle of RSC96 were tested. ADGRG1-R-siRNA-3 transfected into RSC96 cells and its expression was significantly downregulated ($P < 0.05$; Fig. 2A). Based on the results of the data, ADGRG1-R-siRNA-3 was selected for the following experiment. The CCK-8 assay demonstrated that the NF1 cell proliferation was significantly promoted following 24 h, 48 h and 72 h of the treatment with ADGRG1-R-siRNA-3 (24 h,$P < 0.05$, 48 h & 72 h, $P < 0.001$; Fig. 2B). The DNA profiles of S, G1 and G2 phase were examined by using the flow cytometry. According to the Fig. 2C, the cells numbers in S phase were significantly decreased and the cells in G2 phase were significantly increased in the ADGRG1-R-siRNA3 group compared to the control group (Fig. 2C, $p < 0.001$). Meanwhile, the inhibition of the GPR56 slightly decreased the cells numbers in the G1 phase, but without statistical significance. This finding suggested that in RSC96 knockdown GPR56 promoted the progression of the cell cycle.

Effect of ADGRG1-M-siRNA on protein expression of ALP, OPN, OCN and NFAT2 in Osteoblast cells

The results of RT-qPCR showed that the expression of GPR56 coding gene in ADGRG1-M-siRNA-3 group was decreased most significantly, compared with the blank group and the control group ($P < 0.001$), as shown in Fig. 3A. Western blot results also showed that the GPR56 protein expression in ADGRG1-M-siRNA-3 group was lowest than that in the blank group and the control group ($P < 0.01$) (Fig. 3B). Therefore, ADGRG1-M-siRNA3 was selected for the following experiment.

The protein expressions of ALP, OPN, OCN, and NFAT2 in osteoblast MC3T3 were shown in Fig. 3C &3D. The expressions of the osteoblast-specific factors NFAT2 and ALP in ADGRG1-siRNA-3 group were significantly lower than in control and blank group($P < 0.01$ & $P < 0.05$, Fig. 3C&3D), the OCN was also lower than the control and NC group, but there was no statistical significance(Fig. 3D). The protein expression of OPN in ADGRG1-M-siRNA3 treatment group was significantly increased compared with the

control and blank group($P < 0.05$, Fig. 3C). The results suggested that the regulatory effect of GPR56 in MC3T3 cells was mediated by OPN, NFAT2 and ALP.

Effect of ADGRG1-M-siRNA on protein expression of TRAP, OPN, C-Fos and NFAT2 in Osteoclast cells

The results of RT-qPCR and western blot indicated that ADGRG1-M-siRNA-3 had the best knockdown rate of GPR56 on its gene and protein expressions ($P < 0.001$; Fig. 4A and 4B). So, ADGRG1-M-siRNA-3 was selected for the following experiment.

We found that the expressions of C-Fos and NFAT2 in Fig. 4C&4D were significantly decreased in ADGRG1-M-siRNA-3 group compared with the control and blank group ($P < 0.05$&$P < 0.001$), while the expressions of OPN and TRAP showed no significant difference. The results suggested that knockdown GPR56 decreased the expressions of NFAT2 and C-Fos, but did not affect formation of osteoclast.

MC3T3 and Osteoclast were transfected with ADGRG1-M-siRNA-3, the expression of P-Raf1 in Ras signaling pathway

To investigate the protein expression of Ras signaling pathway in MC3T3 and Osteoclast cells, the ADGRG1-M-siRNA-3 was transfected to cells, and Ras, P-Raf1, P-MEK1/2, P-ERK1/2, Raf1, ERK1/2 and MEK1/2 were tested by Western Blot. We discovered that knockdown GPR56, the expression levels of Ras, P-Raf1, P-MEK1/2 and P-ERK1/2 were increased compared with the control group in MC3T3 cells, but there was no statistical significance (Fig. 5A). In osteoclast cells, the expressions of Ras, P-MEK1/2 and P-ERK1/2 had no obvious change after knockdown GPR56, however, the expression of P-Raf1 increased significantly ($P < 0.05$, Fig. 5B). These results suggested that there was no effect in the Ras signaling of GPR56 in osteoblast, whereas in osteoclast the GPR56 can promote the phosphorylation Raf1 protein in Ras signaling pathway.

Discussion

Several therapeutic strategies have been developed for NF1 with scoliosis, including nonoperative and operative methods. Such as abrace, are not effective for dystrophic curves, and early and aggressive surgical intervention is strongly recommended[29]. The surgical options, like fusion and non-fusion techniques, the non-fusion technique(rod) is a better choice for NF1 with scoliosis[30]. Although the growing rod technique is an effective treatment method for early onset spinal deformity, the rapid correction of rigid deformities may increase the risk of neurologic complications. Therefore, more appropriate treatments need to be explored to diagnosis and treat the NF1 with scoliosis. In the present study, we investigated the role of GPR56 in the development of NF1 with scoliosis. GPR56 had been reported to inhibit mesenchymal differentiation and radio resistance in glioblastoma [23]. Also there was

study found that sGPR56 level was also noted in RA patients with higher tumor necrosis factor level[20]. These results illustrated that GPR56 had effect both on cancer and bone disease. Later stages of the Schwann cell lineage have all been implicated as the tumor initiating cell in NF1 associated neurofibromas[31]. Our results discovered that GPR56 inhibited the proliferation and cell cycle progression of NF1 (Fig. 2), which showed that GPR56 was a potential molecule for NF1.

To further validate the role of GPR56 on the scoliosis, we performed osteoblast differentiation and osteoclast cell formation experiments in vitro. We found that after knockdown GPR56, the gene expression levels of osteoblast-specific factors NFAT2 and early markers of osteogenic differentiation ALP were significantly decreased ($P < 0.01$ & $P < 0.05$, Fig. 3C&3D), the gene expression of late markers of osteogenic differentiation OCN was also slightly inhibited (Fig. 3C).However, when knockdown GPR56 the protein expression of OPN was significantly increased($P < 0.05$, Fig. 3C). In osteoclast cells, we found that the expression of osteoclast form marker TRAP in Fig. 4C & 4D was not changed in GPR56 knockdown group, although the expressions of NFAT2 and C-Fos were significantly decreased ($P < 0.05$&$P < 0.001$; Fig. 4C&4D). These analyses indicated that knockdown GPR56 significantly reduced the formation of osteoblasts markers and inhibited the formation of osteoblasts, but had little impact on the markers of osteoclasts, thus broke the dynamic balance between the formation of osteoblasts and the absorption of osteoclasts.

At the same time, in this study, we try to confirm the signaling pathway involved in GPR56-stimulated in osteoblast and osteoclast cells. Previous studies indicated that Ras signaling stimulates proliferation of immature osteoprogenitor cells to increase the number of their osteoblastic descendants in a cell-autonomous fashion[32]. Additionally Ras activation by partial loss of Nf1 gene in osteoprogenitor cells was found to increase proliferation and cause premature apoptosis[33].However, the involvement in GPR56 induction of osteogenic differentiation and osteoclast formation has not been studied. In our study, we found that knockdown GPR56 had no effect on the activation of the Ras pathway in osteoblast cells (Fig. 5A). However, the phosphorylation of P-Raf1, was significantly increased in osteoclast cells by knockdown GPR56 ($P < 0.05$, Fig. 5B), who was a protein in the Ras pathway In summary, the result suggested that there was no effect in the Ras signaling of GPR56 in osteoblast, whereas in osteoclast the GPR56 can affect the P-Raf1 protein in Ras signaling pathway, and knockdown GPR56 broken the formation of osteoblast and the absorption of osteoclast, making to the direction of osteoclast absorption. Above all, the results indicated that GPR56 maybe effect the development and diagnose of NF1 with scoliosis, which was helpful for the further studies on investigating the molecular mechanism of NF1 with scoliosis.

The main limitation in this study was that the signaling pathway factors maybe not enough, which resulted in no statistically significant difference of the GPR56 effect on the osteoblast and osteoclast cells. Then, the research on the molecular mechanism of the disease was not enough, and more in-depth research was needed.

Conclusion

In conclusion, we found that GPR56 may inhibit the proliferation and cell cycle progression of NF1. Furthermore, GPR56 can promote osteoblastic differentiation and osteoclast formation, but had greater influence on osteoblastic differentiation, which indicated that knockdown GPR56 can promote the deterioration of NF1 tumors, meanwhile break the dynamic balance of bone formation and bone absorption, leading to increased bone resorption. This eventually resulted in scoliosis. This study illustrates a possible cause of scoliosis in patients with NF1. The results of our research may have significant enlightenment in understanding the mechanisms of NF1 with scoliosis. And further studies are needed to confirm our findings and to evaluate other possible mechanisms involved.

Abbreviations

neurofibromatosis type 1: NF1; G-protein-coupled receptor: GPR56; magnetic resonance imaging: MRI; Transcriptome sequencing: RNA-seq; computed tomography: CT; CT Mylography: CTM; ADGRG1-Rat-siRNA: ADGRG-R-siRNA; ADGRG1-Mouse-siRNA: ADGRG-M-siRNA

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee and informed consent was obtained from each patient.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors report no conflict of interest

Funding

This study was funded by Beijing Natural Science Foundation (No:7174337) and the Fundamental Research Funds for the Central Universities (No:3332018003).

Author contributions

GX Q and SY C designed the research. SY C and YM Y analyzed data and performed the experiment. GX Q supervised the study. SY C wrote the first draft, YM Y, BH J, ZH W, JG Z, JX S and GX Q made a critical revision of the manuscript for important intellectual content.

Acknowledgements

Not applicable.

References

1. Crump T. Translation of case reports in Ueber die multiplen Fibrome der Haut und ihre Beziehung zu den multiplen Neuromen by F. v. Recklinghausen. Adv Neurol. 1981;29:259–75.
2. Neurofibromatosis. *Conference statement. National Institutes of Health Consensus Development Conference.* Arch Neurol, 1988. **45**(5): p. 575-8.
3. Huson SM, Compston DA, Clark P, Harper PS. A genetic study of von Recklinghausen neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of parental transmission on severity. J Med Genet. 1989;26(11):704–11.
4. Akbarnia BA, Gabriel KR, Beckman E, Chalk D. Prevalence of scoliosis in neurofibromatosis. Spine (Phila Pa 1976). 1992;17(8 Suppl):S244-8.
5. Lyu Q, Zhou C, Song Y, Liu L, Wang L, Zhou Z. Does spinal deformity correction of non-dystrophic scoliosis in neurofibromatosis type I with one-stage posterior pedicle screw technique produce outcomes similar to adolescent idiopathic scoliosis? Spine J. 2017;17(12):1850–8.
6. Vitale MG, Guha A, Skaggs DL. *Orthopaedic manifestations of neurofibromatosis in children: an update*. Clin Orthop Relat Res, 2002(401): p. 107–18.
7. Jain VV, Berry CA, Crawford AH, Emans JB, Sponseller PD. and G. Growing Spine Study, *Growing Rods Are an Effective Fusionless Method of Controlling Early-Onset Scoliosis Associated With Neurofibromatosis Type 1 (NF1): A Multicenter Retrospective Case Series*. J Pediatr Orthop. 2017;37(8):e612–8.
8. Wang Z, Fu C, Leng J, Qu Z, Xu F, Liu Y. Treatment of dystrophic scoliosis in neurofibromatosis Type 1 with one-stage posterior pedicle screw technique. Spine J. 2015;15(4):587–95.
9. Well L, Salamon J, Kaul MG, Farschtschi S, Herrmann J, Geier KI, et al. Differentiation of peripheral nerve sheath tumors in patients with neurofibromatosis type 1 using diffusion-weighted magnetic resonance imaging. Neuro Oncol. 2019;21(4):508–16.
10. Yao Z, Guo D, Li H, Bai Y, Sun B, Zhang X, et al. Surgical Treatment of Dystrophic Scoliosis in Neurofibromatosis Type 1: Outcomes and Complications. Clin Spine Surg. 2019;32(1):E50–5.

11. Gottfried ON, Viskochil DH, Couldwell WT. Neurofibromatosis Type 1 and tumorigenesis: molecular mechanisms and therapeutic implications. Neurosurg Focus. 2010;28(1):E8.
12. Carcavilla A, Santome JL, Pinto I, Sanchez-Pozo J, Guillen-Navarro E, Martin-Frias M, et al. LEOPARD syndrome: a variant of Noonan syndrome strongly associated with hypertrophic cardiomyopathy. Rev Esp Cardiol (Engl Ed). 2013;66(5):350–6.
13. Khong PL, Goh WH, Wong VC, Fung CW, Ooi GC. MR imaging of spinal tumors in children with neurofibromatosis 1. AJR Am J Roentgenol. 2003;180(2):413–7.
14. Quinsey CS, Krause K, Baird LC, Sayama CM, Selden NR. Incidence of symptomatic tethered spinal cord in pediatric patients presenting with neurofibromatosis types 1 and 2. J Neurosurg Pediatr. 2018;21(5):456–9.
15. Thakkar SD, Feigen U, Mautner VF. Spinal tumours in neurofibromatosis type 1: an MRI study of frequency, multiplicity and variety. Neuroradiology. 1999;41(9):625–9.
16. Redmond D, Poran A, Elemento O. Single-cell TCRseq: paired recovery of entire T-cell alpha and beta chain transcripts in T-cell receptors from single-cell RNAsEq. Genome Med. 2016;8(1):80.
17. Oncu-Oner T, Unalp A, Porsuk-Doru I, Agilkaya S, Guleryuz H, Sarac A, et al. GPR56 homozygous nonsense mutation p.R271* associated with phenotypic variability in bilateral frontoparietal polymicrogyria. Turk J Pediatr. 2018;60(3):229–37.
18. Stevenson DA, Yan J, He Y, Li H, Liu Y, Zhang Q, et al. Multiple increased osteoclast functions in individuals with neurofibromatosis type 1. Am J Med Genet A. 2011;155A(5):1050–9.
19. Xu L, Begum S, Hearn JD, Hynes RO. GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. Proc Natl Acad Sci U S A. 2006;103(24):9023–8.
20. Tseng WY, Jan Wu YJ, Yang TY, Chiang NY, Tsai WP, Gordon S, et al. High levels of soluble GPR56/ADGRG1 are associated with positive rheumatoid factor and elevated tumor necrosis factor in patients with rheumatoid arthritis. J Microbiol Immunol Infect. 2018;51(4):485–91.
21. Chen Z, Gao P, Li Z. Expression of G Protein-coupled Receptor 56 Is an Unfavorable Prognostic Factor in Osteosarcoma Patients. Tohoku J Exp Med. 2016;239(3):203–11.
22. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, et al. Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. J Clin Endocrinol Metab. 2001;86(3):1274–80.
23. Moreno M, Pedrosa L, Pare L, Pineda E, Bejarano L, Martinez J, et al. GPR56/ADGRG1 Inhibits Mesenchymal Differentiation and Radioresistance in Glioblastoma. Cell Rep. 2017;21(8):2183–97.
24. Gao M, Zhu B, Xu Z, Liu S, Liu J, Zhang G, et al. Association between acromegaly and a single nucleotide polymorphism (rs2854744) in the IGFBP3 gene. BMC Med Genet. 2018;19(1):182.
25. Giera S, Luo R, Ying Y, Ackerman SD, Jeong SJ, Stoveken HM, et al., *Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells*. Elife, 2018. 7.

26. Wang W, Wu BQ, Chen GB, Zhou Y, Li ZH, Zhang JL, et al. Meta-analysis of the association of IGFBP3 and IGF1 polymorphisms with susceptibility to colorectal cancer. Neoplasma. 2018;65(6):855–64.
27. Tang W, Chen S, Liu J, Liu C, Wang Y, Kang M. Investigation of IGF1, IGF2BP2, and IGFBP3 variants with lymph node status and esophagogastric junction adenocarcinoma risk. J Cell Biochem. 2019;120(4):5510–8.
28. Vella V, Nicolosi ML, Cantafio P, Massimino M, Lappano R, Vigneri P, et al. DDR1 regulates thyroid cancer cell differentiation via IGF-2/IR-A autocrine signaling loop. Endocr Relat Cancer. 2019;26(1):197–214.
29. Funasaki H, Winter RB, Lonstein JB, Denis F. Pathophysiology of spinal deformities in neurofibromatosis. An analysis of seventy-one patients who had curves associated with dystrophic changes. J Bone Joint Surg Am. 1994;76(5):692–700.
30. Kim HW, Weinstein SL. Spine update. The management of scoliosis in neurofibromatosis. Spine (Phila Pa 1976). 1997;22(23):2770–6.
31. Jessen KR, Mirsky R. Schwann Cell Precursors; Multipotent Glial Cells in Embryonic Nerves. Front Mol Neurosci. 2019;12:69.
32. Papaioannou G, Mirzamohammadi F, Kobayashi T. Ras signaling regulates osteoprogenitor cell proliferation and bone formation. Cell Death Dis. 2016;7(10):e2405.
33. Yu X, Chen S, Potter OL, Murthy SM, Li J, Pulcini JM, et al. Neurofibromin and its inactivation of Ras are prerequisites for osteoblast functioning. Bone. 2005;36(5):793–802.
34. Tsuda H, Zhao N, Imai K, Ochiai K, Yang P, Suzuki N. BIX01294 suppresses osteoclast differentiation on mouse macrophage-like Raw264.7 cells. Bosn J Basic Med Sci. 2013;13(4):271–5.

Figures

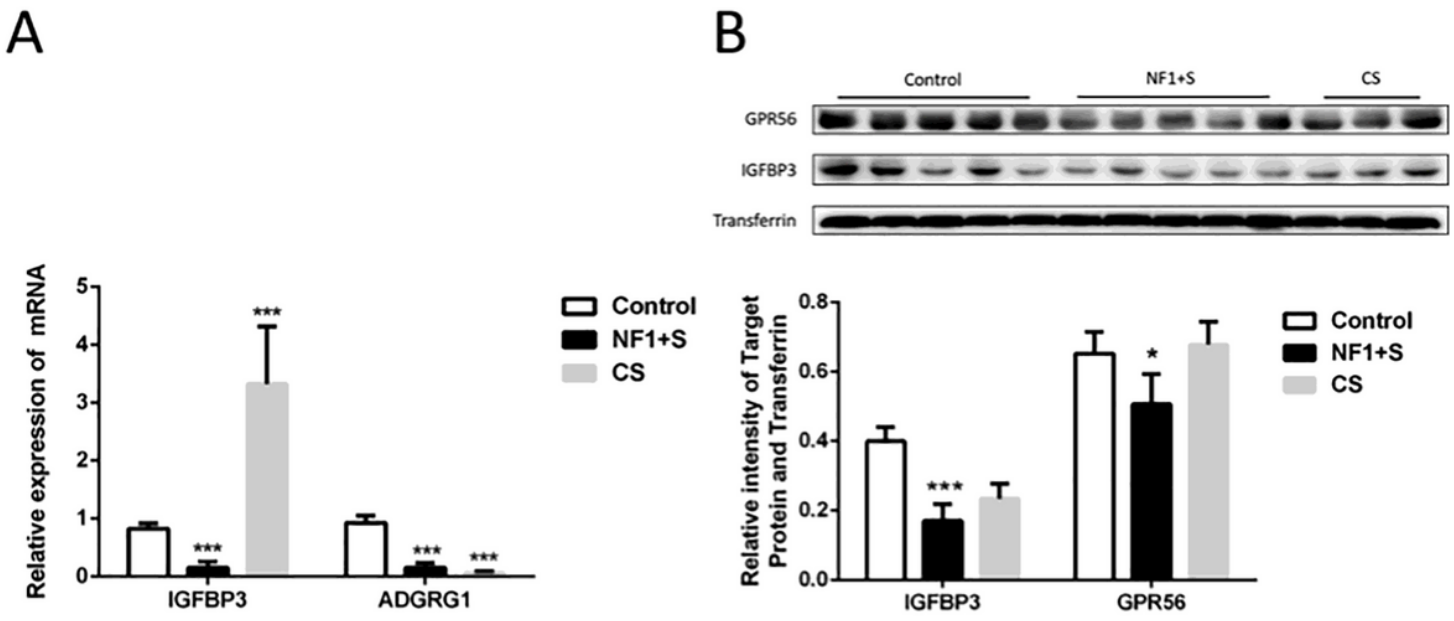

Figure 1

The expression of ADGRG1 and IGFBP3 in blood of NF1with scoliosis A. The relative mRNA expression of ADGRG1 and IGFBP3 by PCR B. The protein expression of ADGRG1 and IGFBP3 by WB
*,P<0.05;**,P<0.01;***/****,P<0.001

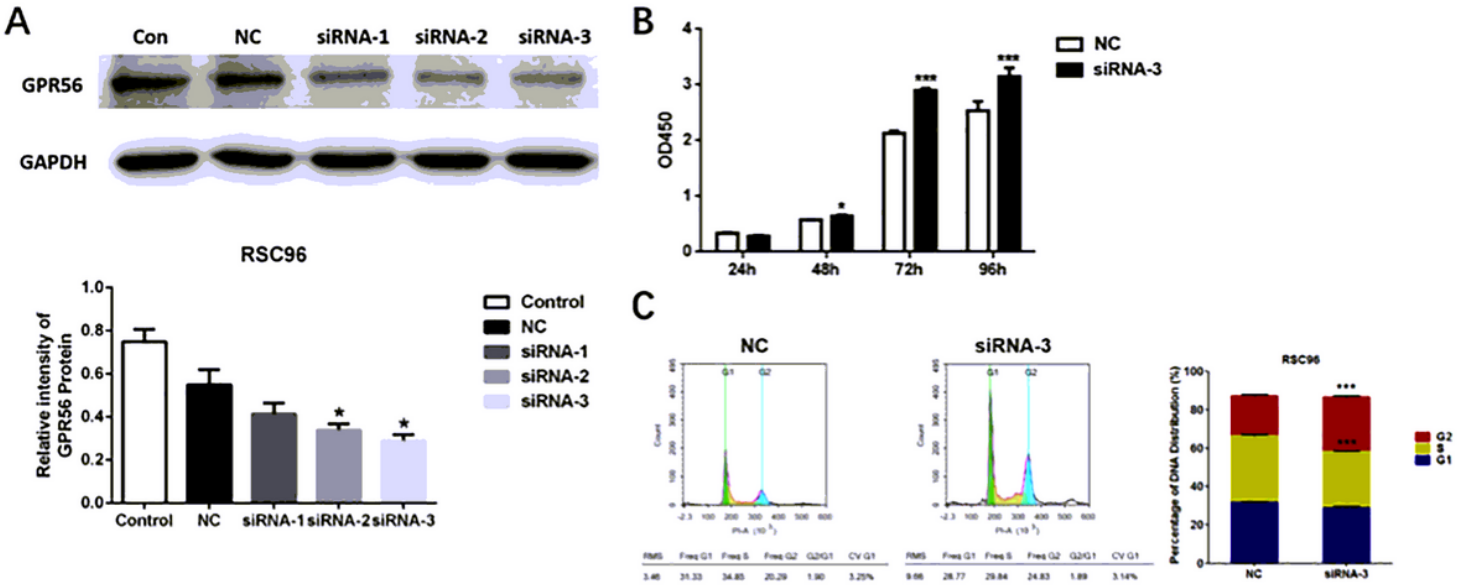

Figure 2

The effects of GPR56 on NF1 cells. A . The expression of GPR56 by WB. B. Effect of GPR56 on the proliferation of NF1 cells by CCK-8. C. Effect of GPR56 on the circle of NF1 cells by Flow Cytometer
*,P<0.05;**,P<0.01;***/****,P<0.001

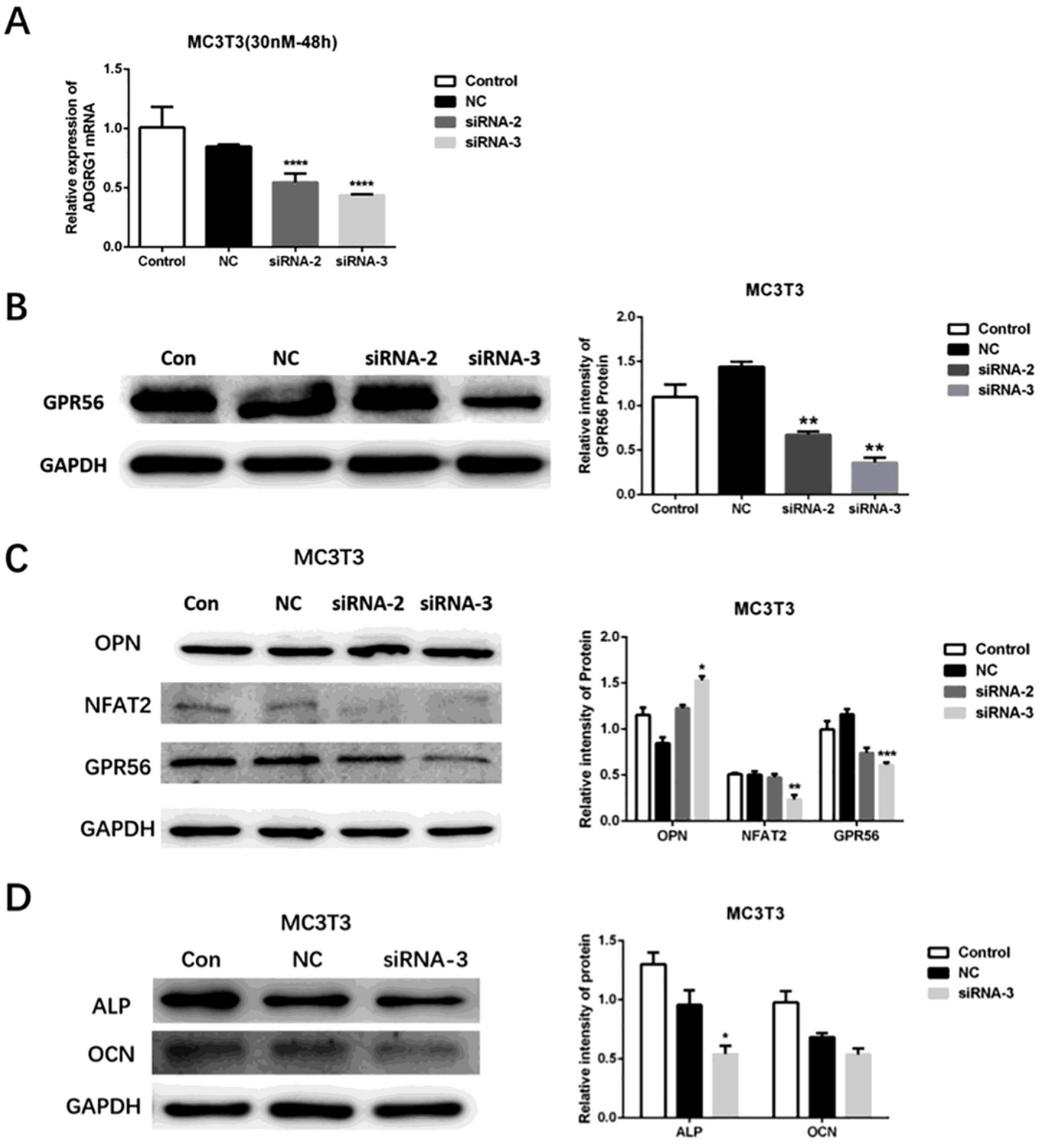

Figure 3

The effects of GPR56 on osteoblast. A&B . The expression of GPR56 by PCR and WB. C&D. The expression of GPR56, OPN, NFAT2, ALP and OCN by WB. *,P<0.05;**,P<0.01;***/****,P<0.001

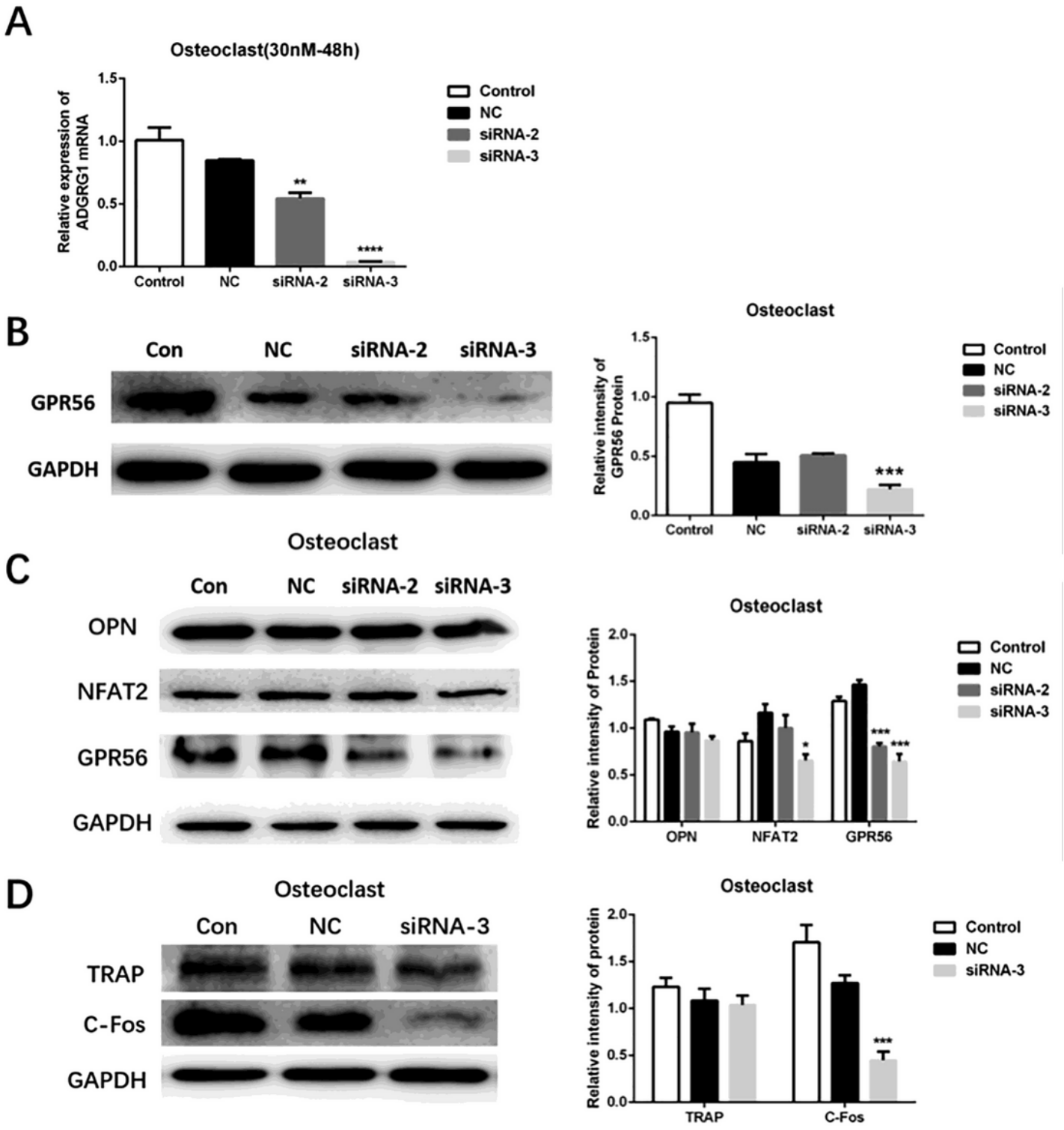

Figure 4

The effects of GPR56 on osteoclast. A&B. The expression of GPR56 by PCR and WB. C&D. The expression of GPR56, OPN, NFAT2, TRAP and C-Fos by WB. *,P<0.05;**,P<0.01;***/****,P<0.001

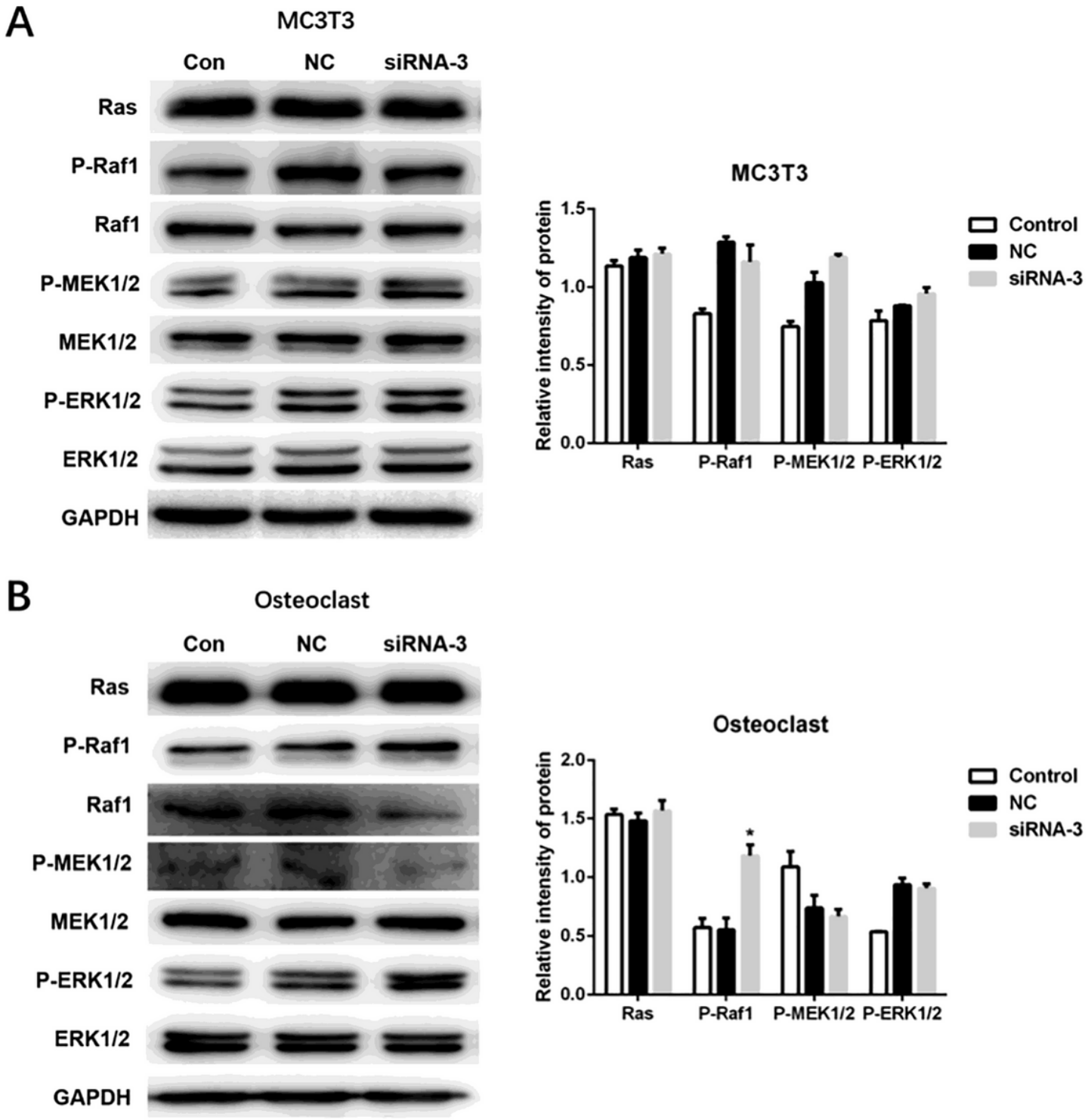

Figure 5

The pathway of GPR56 in osteoblast and osteoclast A. the signaling proteins P-Raf1, P-MEK1/2, P-ERK1/2 expression in osteoblast B. the signaling proteins P-Raf1, P-MEK1/2, P-ERK1/2, expression in osteoclast. *,P<0.05;**,P<0.01;***/****,P<0.001