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ROCK Inhibition by GSK429286A Enhance Osteoblast Differentiation and Mineralization of Human Skeletal (Mesenchymal) Stem Cells

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Abstract:
Background: Multiple signaling pathways have been found to regulate the osteoblast differentiation of human bone marrow stromal (skeletal) stem cells (hBMSCs) including ROCK signaling pathway. ROCK1 and ROCK2 are implicated in various cellular activities involving cellular proliferation, apoptosis, cell adhesion, motility, extracellular matrix remodeling, actin cytoskeleton organization, and contraction of smooth muscles. However, the crucial role of ROCK signaling during osteoblast differentiation of hBMSCs, is not studied in details. Methods: A ROCK1/2 inhibitor, GSK429286A, identified during a chemical biology screen of a small molecule library for their effect on osteoblast differentiation of hBMSCs. Alkaline phosphatase activity and staining tests, indicators for osteoblastic differentiation and Alizarin red staining, indicator for in vitro formation of mineralized matrix, were performed. Changes in gene expression were assessed using qRT-PCR. Results: In vitro treatment of hMSCs with GSK429286A (3μM) led to significant increase in osteoblast differentiation as demonstrated by elevated activity of the ALP, formation of mineralized matrix in vitro and upregulation of osteoblast-related expression of genes including ALP, OC, ON, and OPN. To determine the molecular mechanisms, we examined the effects of GSK429286A on a molecular signature of a number of gene targets known to affect hBMSCs differentiation into osteoblasts. GSK429286A upregulated gene expression of LIF, SOCS3, RRAD, NOTCH3, TNF, CXCL1, VDR, IL6, and THBS2, which are targets of a number of signaling pathways e.g. TGFβ, insulin, focal adhesion, Notch, Vitamin D and cytokine signaling. Conclusions: We identified a ROCK1/2 inhibitor (GSK429286A) as a positive regulator of hBMSC osteoblastic distinction which could be beneficial as a therapeutic alternative for managing bones diseases characterized by impaired bone formation.

Keywords: ROCK Signaling; ROCK1/2 Inhibition; GSK429286A; human bone marrow stromal (skeletal) stem cells; osteoblast differentiation; in vitro mineralization

INTRODUCTION:

Rho kinases ROCK1 and ROCK2 are serine/threonine kinases which serve as downstream effectors for the RhoA, RhoB, and RhoC small GTPases [1,2]. ROCK1 and ROCK2 contain 1354 and 1388 amino acids, respectively, where they share 65% overall amino acid sequence identity [1,3], around 92% in the kinase domain, while they are different within their coiled-coil domains with 55% similarity [1]. Both ROCKs have the N-terminal kinase domain, the coiled-

coil domain containing the Rho-binding domain [4], and the Pleckstrin homology domain C-terminal [5]. ROCKs are implicated in various cellular activities, involving cellular proliferation, apoptosis, extracellular matrix remodeling, motility, cell adhesion, actin cytoskeleton organization, and contraction of smooth muscles [1,6,7]. In general, both ROCK1 and ROCK2 have similar roles. However, some environmental factors such as their activation and subcellular localization, can affect their cellular function.

In the bone marrow, multipotent stem cells known as human bone marrow skeletal (mesenchymal) stem cells (hBMSCs) could distinguish into a number of mesodermal cells, which include osteoblasts [8,9]. Several signaling pathways have been found to regulate the osteoblast differentiation of hBMSCs including ROCK [2,10], JAK/STAT [11], Wnt/β-catenin [12], TGFβ [13], focal adhesion [14], Notch Signaling [15], and Hh signaling [16]. Nevertheless, their specific role in the differentiation of osteoblasts stayed to be determined.

Small-molecule inhibitors of particular signaling pathways have recently been used as a powerful tool in vitro to unveil the molecular mechanisms regulating the osteoblast differentiation of stem cell differentiation [11,17,18]. Thru a small molecule library monitor, we discovered GSK429286A, a ROCK1/2 inhibitor, to be an enhancer of hBMSC osteoblastic differentiation and may suggest that pharmacological inhibition of ROCK signaling with a small molecule inhibitor may be employed in regenerative medicine to increase bone formation.

MATERIALS AND METHODS

Culturing of cells

As a model for hBMSCs, the hBMSC-TERT cell line had been utilized in all of the experiments in this research. The cell line was kindly given by Prof. Moustapha Kassem, Molecular Endocrinology Unit (KMEB), University Hospital of Odense and University of Southern Denmark. The hBMSC-TERT line was generated by overexpressing the human telomerase reverse transcriptase gene (hTERT). hBMSC-TERT has the same unlimited self-renewal, potency, and gene expression profile as primary hBMSCs [19,20].

As previously mentioned, Dulbecco modified eagle medium (DMEM), which is a basal medium complemented by 4 mM L-glutamine, 4,500 mg/l D-glucose, and 110 mg/l 10% sodium pyruvate, as well as 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% non-essential amino acids, has been kept [19]. Thermo Fisher Scientific Life Sciences, Waltham, MA (http://www.thermofisher.com) provided all of the reagents. At 37°C and 95 % humidity, cells had been incubated in incubators containing 5% CO2.

Differentiation of osteoblasts

When the cells reached 80%–90% confluence, the medium was substituted with osteoblast induction medium (DMEM comprising 10% FBS, 1% penicillin-streptomycin, 50 mg/ml L-ascorbic acid (Wako Chemicals GmbH, Neuss, Germany, http://www.wako-chemicals. de/), 10 mM b-glycerophosphate (Sigma-Aldrich), 10 nM calcitriol (1a,25-dihydroxyvitamin D3; Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich) as previously described in [11]. GSK429286A was bought from Selleckchem Inc. (Houston, TX, http://www.selleckchem.com), which also has a stem cell signaling small molecule inhibitor library. The osteoblast induction

medium was treated with small molecule inhibitors at a concentration of 3μM, and the cells were exposed to the inhibitor continuously during the differentiation process. Osteoblast induction medium with dimethyl sulfoxide (DMSO) as a vehicle was used to culture control cells.

Assay of cell viability

As previously described in [11], the alamarBlue assay has been used to perform cell viability assays as per the manufacturer's instructions (Thermo Fisher Scientific). Cells have been cultivated in 96-well plates in 300 μl of medium in the existence of 0.3, 3, and 30 μM GSK429286A in contrast to DMSO vehicle-treated control cells for the dose-response growth curve. On days 1, 2, and 3, 30 μl of alamarBlue substrate (10%) became applied to each well, and the plates were incubated for 1 hour at 37°C in the darkness. A BioTek Synergy II microplate reader in fluorescent mode was used to take the readings (Ex 530 nm/Em 590 nm) from BioTek Inc. in Winooski, VT, USA. Cells have been cultivated in 96-well plates at 300 μl of the medium for cell viability. On day10, 30μl of alamarBlue substrate (10%) became applied to each well, and plates have been incubated for 1 hr at 37°C in the dark. The BioTek Synergy II microplate reader (BioTek Inc., Winooski, VT, US) has been utilized to obtain readings in fluorescent mode (Ex 530 nm/Em 590 nm).

Apoptosis measurement

After exposure of the cells to GSK429286A (3 μM), a fluorescence-based apoptosis assay that use the acridine orange/ethidium bromide (AO/EtBr) staining process has been conducted as earlier mentioned [21] relative to DMSO-vehicle treated control cells. Cells with dual fluorescent staining solution (1.0 μl) comprising 100 μg/ml AO and 100 μg/ml EtBr (AO/EB, Sigma, St. Louis, MO, USA) have been stained on Day 3. Before being imaged with a Nikon Eclipse Ti fluorescence microscope, cells have been combined with AO/EtBr (1:100) dye solution for one minute (Nikon, Tokyo, Japan).

Quantification of activity of alkaline phosphatase

BioVision ALP activity colorimetric assay kit (BioVision, Inc., Milpitas, CA, http://www.biovision.com/) has been utilized to measure alkaline phosphatase (ALP) activity with some modifications, as mentioned previously in [11]. 96-well plates were used to culture the cells. On day 10 of osteoblast differentiation, the cells have been rinsed once with PBS and fixed for 30 sec at room temp with 3.7 % formaldehyde in 90 % ethanol. After removal of the fixative, 50 μl of p-nitrophenyl phosphate solution has been applied per each well and incubated for 30–60 minutes. A SpectraMax/M5 fluorescence spectrophotometer plate reader was used to calculate optical densities at 405 nm, and the activity of the ALP enzyme was then normalized to cell number.

Staining of alkaline phosphatase

In osteoblast differentiation medium, cells were cultivated in a 6-well plate. On day 10, alkaline phosphatase staining was performed as previously described in [11]. After washing the cells in PBS, they were fixed in a 10 mM acetone/citrate buffer at pH 4.2 for five minutes at room temp. After removing the fixative, Naphthol /Fast Red stain was applied for 1 hour at room temp [0.2 mg/mL Naphthol AS-TR phosphate substrate (Sigma)] [0.417 mg/mL Fast Red (Sigma)]. After that, the cells have been washed three times with water and images have been captured under a microscope.

Alizarin Red S Staining for the formation of mineralized matrix

As previously described in [11], alizarin red staining has been conducted on day 21 of osteoblast differentiation. The cells have been washed twice with PBS before being fixed for 10 minutes at room temp with 4% paraformaldehyde. The fixative has been rinsed , and the cells have been therefore washed three times with distilled water before being stained for 10–20 minutes at room temp with the 2 % Alizarin Red S Staining Kit (ScienceCell, Research Laboratories, Cat. No.0223).The cells have been then washed in water and images have been obtained using a microscope.

Extraction of RNA and synthesis of cDNA

The total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada, https://norgenbiotek.com/) was used to extract total RNA from cell pellets on day 10 of osteoblast differentiation, as mentioned earlier [11]. NanoDrop 2000 was used to measure the concentrations of total RNA isolated (ThermoFisher Scientific Life Sciences). The High Capacity cDNA Transcription Kit (ThermoFisher Scientific Life Sciences) was used to synthesize cDNA from 500 ng of total RNA, in compliance with the manufacturer's guidelines.

Quantitative Real Time- Polymerase Chain Reaction

The Applied Biosystems ViiA™ 7 Real-Time PCR System was used to conduct quantitative (RT-PCR) utilizing fast SYBR Green (ThermoFisher Scientific Life Sciences). Table 1 lists the primers that were used in this research. The relative expression was measured utilizing the 2ΔCTvalue approach, and the analysis was carried out as mentioned earlier [22].

Table 1: List of SYBR Green primers used in this research

Gene Name	Forward Primer	Reverse Primer
ACTB	5′AGCCATGTACGTTGCTA	5′AGTCCGCCTAGAAGCA
ALPL	5’ GGA ACT CCT GAC CCT TGA CC 3’	5’ TCC TGT TCA GCT CGT ACT GC 3’
OC	GGCAGCGAGGTAGTGAAGAG	CTCACACACCTCCCTCCTG
ON	5′GAGGAAACCGAAGAGGAGG3′	5′GGGGTGTTGTTCTCATCCAG3′
RUNX2	5′GTAGATGGACCTCGGGAACC3′	5′GAGGCGGTCAGAGAACAAAC3′
OPN	GGTGATGTCCTCGTCTGTA	CCAAGTAAGTCCAACGAAAG
ROCK1	AAAATTGTGTGAGGAGGACATGG	TTCATCCCAACATTCTTGGATCT
ROCK2	GCAATGCGGTAAAAAGCGA	GGGAATCATGGTGTGACCAA
LIF	5`GCCACCCATGTCACAACAAC	5`CCCCCTGGGCTGTGTAATAG
SOCS3	5′TTCGGGACCAGCCCCC3′	5′AAACTTGCTGTGGGTGACCA3′
RRAD	5′GCGGAAACCCTAAAGTCCGA	5′GTCCGGGACCGTCCACT
NOTCH3	5′CCTGTGGCCCTCATGGTATC	5′CATGGGTTGGGGTCACAGTC
TNF	5′ACTTTGGAGTGATCGGCC3′	5′GCTTGAGGGTTTGCTACAAC3′
CXCL1	5′CCAGCTCTTCCGCTCCTC3′	5′CACGGACGCTCCTGCTG3′
VDR	CTCTGATAGCCTCATGCCAGG	ACCCAAAGGCTTCCCAAAGAG
IL6	CGAGCCCACCGGGAACGAAA	GGACCGAAGGCGTTGTGGAG
THBS2	5′TTGGCAAACCAGGAGCTCAG3′	5′GGTCTTGCGGTTGATGTTGC3′

Statistical analysis

Microsoft Excel 2010 and GraphPad Prism 6.0 program (GraphPad, San Diego, CA, USA) have been used for statistical analysis and graphing, respectively. Data from at least two independent

experiments were shown to be mean ± SEM. An unpaired, two-tailed Student t-test was used to determine statistical significance, with p-values < 0.05 deemed statistically significant.

RESULTS

Effects on hBMSC cell proliferation

Using ALP activity quantification as a read-out, we previously reported the screening findings of a small molecule library which defined a group of small molecule inhibitors with various impacts on osteoblast differentiation of hBMSCs [11]. Among these, GSK429286A, a ROCK1/2 inhibitor, exhibited potent enhancing effects. Thus, we studied GSK429286A in a comprehensive manner. We first assessed the effect of in vitro treatment of GSK429286A at increasing concentrations (range: 0.3-30 µM) for 1,2, and 3 days on hBMSCs cell proliferation (Fig.1a). No significant effects of GSK429286A on hBMSCs cell proliferation were observed at dose of 0.3 and 3 µM. However, GSK429286A inhibited hBMSCs cell proliferation at dose of 30 µM following 3 days treatment. To determine the cellular mechanism, we performed apoptosis assay following GSK429286A treatment (3 µM) for 3 days but no significant changes were detected in apoptotic cells compared to DMSO-vehicle treated control cells (Fig. 1b).

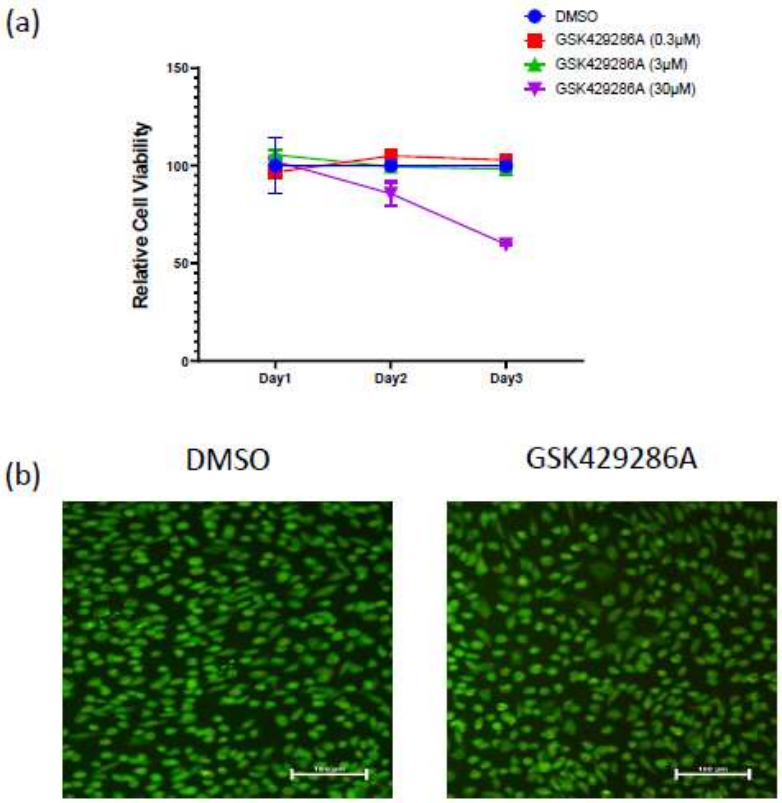

Figure 1: Effects of GSK429286A therapy on the viability of hBMSCs. (a) The graph depicts the dose-response proliferation curve of hBMSCs in response to various doses of GSK429286A therapy against DMSO-treated control cells, as calculated by cell viability over three days. DMSO: dimethyl sulfoxide. (b) On the third day following exposure, representative fluorescence images of GSK429286A-treated hBMSCs (3.0 µM) against DMSO-treated control cells. Magnification of photomicrographs 20x. Apoptotic (cells with green condensed chromatin) and necrotic cells were detected using AO/EtBr staining (red). DMSO: dimethyl sulfoxide.

Effects on osteoblast differentiation of hBMSCs

GSK429286A-treated hBMSCs (3 µM) exhibited a significant enhancement in ALP production as confirmed by increased cyochemical staining intensity (Fig. 2a), which was mirrored by the increase in ALP activity measurement at day 10 post-osteoblast differentiation induction (Fig. 2b). At day 10 post-osteoblast differentiation induction, GSK429286A had no impact on hBMSC viability (Fig. 2c).

GSK429286A-treated hBMSCs (3 µM) showed a significant increase in the formation of mineralized matrix as demonstrated by Alizarin red staining (Fig. 3a), that was correlated to a large upregulation in the expression of many osteoblast gene markers: ALP, OC, ON, and OPN (Fig. 3b).

GSK429286A was given to hBMSCs at the same concentration (3 µM) to verify that it targets the ROCK signaling pathway, and the expression of ROCK1 and ROCK2 was evaluated utilizing qRT-PCR 48 hours later. The ROCK signaling pathway was significantly inhibited by GSK429286A, as shown in Fig. 3c.

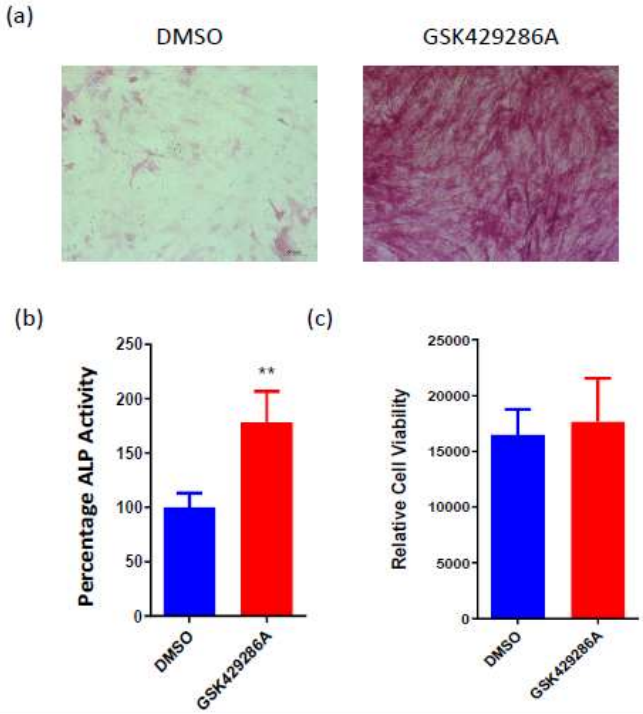

Figure 2: Effects of GSK429286A therapy on the differentiation of osteoblasts with hBMSCs. (a) On Day 10, post-osteoblastic differentiation, indicative alkaline phosphatase (ALP) staining of GSK429286A-treated hBMSCs (3.0 µM) against DMSO-treated control cells. Magnification of photomicrographs 10x. ALP, alkaline phosphatase; DMSO, dimethyl sulfoxide. (b) On day 10 post-osteoblastic differentiations, ALP activity in GSK429286A-treated hBMSCs (3.0 µM) against DMSO-treated control cells was quantified. The data is provided as mean percentage ALP activity ±SEM (n=20). (c) On day 10 post-osteoblastic differentiation, Alamar Blue assay was used to compare cell viability in GSK429286A-treated hBMSCs (3.0 µM) to DMSO-treated control cells. The data is provided as mean ± SEM (n = 20). DMSO: dimethyl sulfoxide.

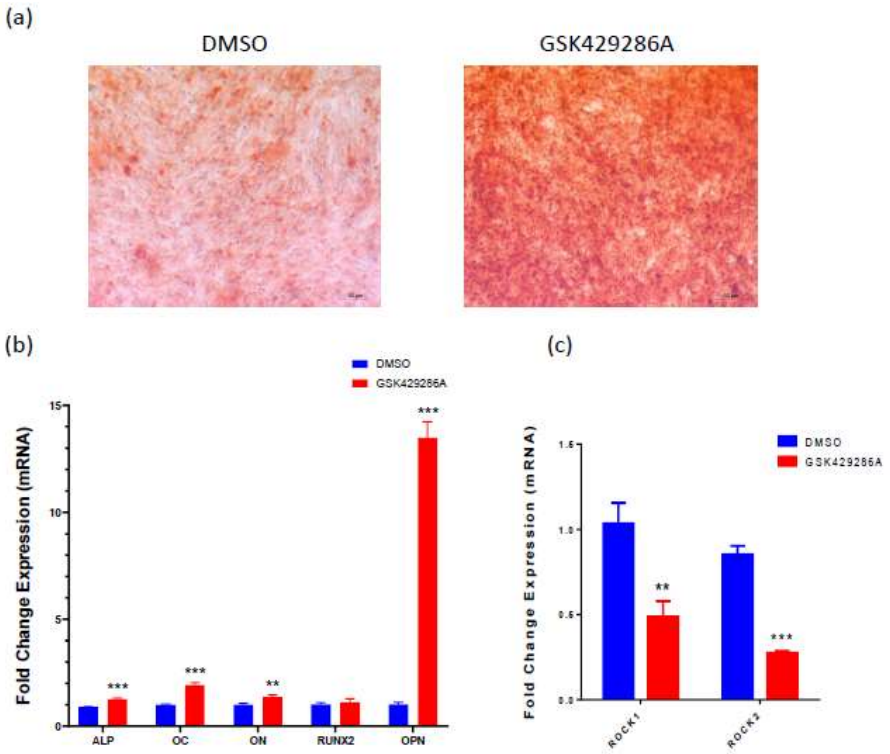

Figure 3. Effects of GSK429286A treatment on hBMSCs function in vitro. (a) On day 21 post-osteoblastic differentiation, cytochemical staining for mineralized matrix formation utilizing Alizarin red in the presence (right panel) or absence (left panel) of GSK429286A (3.0 μM). Magnification of photomicrographs 10x. (b) In the presence (red) or absence (blue) of GSK429286A (3.0 μM), quantitative RT-PCR analysis for expression of genes of ALP, OC, ON, RUNX2, and OPN in hBMSCs on day 10 post osteoblast differentiation. (c) Quantitative RT-PCR analysis for expression of genes of ROCK1 and ROCK2 in hBMSCs treated with GSK429286A (3.0 μM) for 48 hours. Expression of gene has been normalized to β-actin. Results from two separate experiments were viewed as mean fold shift ± SEM (n = 6); *p < 0.05; *** p ≤ 0.0005. ALP, alkaline phosphatase; OC, Osteocalcin; ON, Osteonectin; RUNX2, runt-related transcription factor 2; OPN, Osteopontin; DMSO, dimethyl sulfoxide.

GSK429286A affects multiple signaling pathways during osteoblast differentiation of hBMSCs

Previously, we mentioned a number of small molecule inhibitors with different effects on osteoblast differentiation of hBMSCs as assessed by global gene expression profiling [11,15,16]. A number of significantly enriched signaling pathways known to control osteoblast differentiation of hBMSCs were identified including TGFβ signaling, insulin signaling, focal adhesion, Notch Signaling, Vitamin D signaling, IL-6 signaling, endochondral ossification, TNF alpha, and cytokines and inflammatory response. Thus, we decided to evaluate the effects of GSK429286A on these signaling pathways by assessing gene expression of their respective gene signature (Fig. 4). Gene expressions of LIF, SOCS3, RRAD, NOTCH3, TNF, CXCL1, VDR, IL6, and THBS2 showed significant increase in GSK429286A-treated hBMSCs (3 μM) relative to DMSO vehicle-treated osteoblast differentiation control cells at day 10.

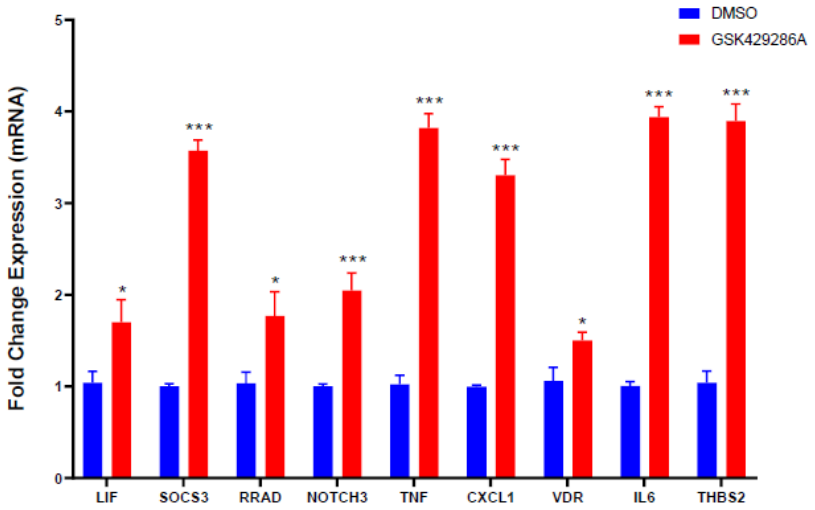

Figure 4. GSK429286A enhances expression of multiple osteoblast-associated genes. Quantitative RT-PCR analysis for a selected panel of osteoblast differentiation-associated genes in GSK429286A-treated hBMSCs aganist DMSO-treated control using qRT-PCR on day 10 post osteoblasts differentiation in the presence (red) or absence (blue) of GSK429286A (3.0 μM). Expression of genes was normalized to β-actin. Results from two independent experiments were viewed with mean fold shift ± SEM (n=6); *$p < 0.05$; ***$p \leq 0.0005$.

DISCUSSION

hBMSCs are multipotent stem cells that can give rise to bone-forming osteoblastic cells during bone metabolism [8,9]. Disclosing the signaling pathways involved in osteoblast differentiation, knowledge of bone disease pathogenesis is crucial. [23,24]. Small molecule inhibitors targeting particular intracellular signaling pathways are being commonly used in vitro as a substitute for conventional cellular differentiation procedures which involve a complex combination of growth factors and cytokines [11,17]. During a small molecule library functional screen, we discovered GSK429286A to be an enhancer of hBMSC differentiation to osteoblastic cells in the present research [11].

GSK429286A is a small molecule ROCK1/2 inhibitor. ROCK1 and ROCK2 are essential regulators for cellular cytoskeleton, form, and motility, in addition to cellular proliferation and apoptosis [1,6,25]. ROCK1 and ROCK2 are usually described as oncogenes [6,26], and their upregulation has been frequently associated with poor prognosis in cancer studies such as osteosarcoma, gastric and laryngeal squamous cell carcinoma [6,26-30]. Moreover, increased ROCK signaling has been highly correlated with the pathogenesis of cardiovascular diseases like cardiac fibrosis and cardiac hypertrophy, pulmonary hypertension, and heart failure [1]. Thus, suppressing its activity has been arising as a target for the treatment of such diseases.

We observed that GSK429286A treatment enhanced osteoblast differentiation in vitro. Recent studies have reported a critical role of ROCK signaling in bone metabolism [2,6,10,31-33], which is concordant with our data.

A research was conducted to examine the impact of a ROCK inhibitor, fasudil hydrochloride, on stromal cell lines reported a significant increase in the expression of collagen-I, OC, and BMP-2, a significant stimulation of ALP activity, and an increased mineralization, which suggest an enhancement effect of fasudil on osteoblastic differentiation of stromal cell lines [32]. Another study also reported that inhibition of ROCK with Fasudil and Y-27632, enhances both osteoclastogenesis and osteoblastogenesis, and that ROCK inhibitors administered locally improve rat calvarial defect bone healing [34]. Moreover, ROCK inhibition has been shown to induce ectopic bone formation [35]. Prowse et al. recorded that inhibition of ROCK signaling with Y-27632 significantly increases osteoblast differentiation and biomineralization [31]. In Rat Calvarial Osteoblasts, ROCK inhibition significantly increased ALP and OC mRNA levels, bone sialoprotein expression, and nuclear translocation of Runx2, which was mirrored by increases in mineralization of the cultured osteoblast [31,33,36].

Global gene expression profiling of hBMSCs following treatment with previously reported small molecules to inhibit osteoblastic differentiation [11,15,16] revealed significant changes in many osteoblast differentiation-associated intracellular signaling pathways including TGFβ signaling [13], insulin signaling [37], focal adhesion [14], Notch Signaling [15], Vitamin D signaling [38], IL-6 signaling [39], endochondral ossification [40], TNF [41], and cytokines and inflammatory response [42]. Hence, in the current research, we studied the impact of GSK429286A on the reported intracellular signaling pathways by examining their signature gene which showed significant elevation in the expressions of LIF, SOCS3, RRAD, NOTCH3, TNF, CXCL1, VDR, IL6, and THBS2 suggesting that treatment with GSK429286A may activate a number of osteoblast differentiation-associated signaling pathways, secondary to its impact on ROCK signaling, and that osteoblastic differentiation of hBMSCs involves a coordination of various signaling pathways.

ROCK inhibition efficiency in improving and increasing osteoblast differentiation and mineralization has many advantages in bone tissue engineering applications [31]. Thus, ROCK inhibitor could be considered as a supplement in standard osteoblast differentiation protocol and may be considered for in vivo targeting of hBMSC with the aim of enhancing bone formation in regenerative medicine protocols.

List of abbreviations:
hBMSCs: human bone marrow stromal (skeletal) stem cells; ROCK: Rho-associated protein kinase; hTERT: Human telomerase reverse transcriptase; qRT-PCR: Quantitative reverse transcriptase-polymerase chain reaction; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; PBS: Phosphate-buffered saline; ALP: Alkaline phosphatase; OC, Osteocalcin; ON, Osteonectin; RUNX2, runt-related transcription factor 2; OPN, Osteopontin; ALZR: Alizarin red

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Conflict of interest
No conflicts of interest have been declared by the researcher.

Declarations

Consent to publication: Not applicable.

Availability of data and material: Data can be accessed on request.

Competing interests: There are no competing interests declared by the researcher.

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Authors' contributions: N.A. carried out design, conception, performing experiments, data analysis, and writing manuscripts.

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