RESEARCH
O&G Forum 2024; 34 – 2s: 390 - 395

INVESTIGATING THE ROLE OF RUTIN ON REGULATION OF MIRNA TARGETING RUNX2

Running title: Investigating the role of Rutin on regulation of miRNA targeting Runx2
Type of study: Original study

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Abstract
Introduction: The cells called osteoblasts are responsible for maintaining and repairing bone, and Runx2 is essential for osteoblast differentiation, function, and ultimately bone biology. MiRNAs are now regarded as one of the most effective regulators at the post-transcriptional level of gene expression, especially in light of the emergence of epigenesis and related regulatory mechanisms. Rutin can stimulate cell proliferation and osteogenic differentiation in vitro, which can effectively prevent and treat osteoporosis, according to research
Materials and Methods: Real time PCR, Western blot, Statistical analysis
Result: Rutin upregulates the expression of Runx2 and suppress the expression of miR-889-5p mRNA, there by promoting osteoblast differentiation and the related genes to be expressed in the osteoblast.
Conclusion: Rutin was able to promote osteoblastic differentiation by upregulating Runx2 and by directly reducing the level of microRNA 889 5p.
KEYWORDS:
Rutin, Runx2, osteoblast differentiation, osteoblast, miRNA, hsa-miR-889-5p, osteogenic medium

INTRODUCTION:

Levanon et al. (1994) (1)isolated and characterised runx transcription factors (runt-related transcription factors), which are lineage-specific transcription factors required for hematopoietic (Runx1/AML1), neuronal, gastrointestinal (Runx3/AML2), and osteogenic cell differentiation (Runx2/AML3). The runt domain, a 128-amino acid highly conserved DNA-binding domain, is the defining feature of this family, which shares similarity with the Drosophila segmentation gene product runt (Ogawa et al., 1993) (2)When looking for transcription factors exclusive to osteoblasts, Runx2, also known as Cbfa1/AML3/Pebp2alphaA, was found by examining the regulation of the expression of osteocalcin, the only gene expressed in osteoblasts and no other extracellular matrix (ECM)-producing cell type. The runx2 gene has eight exons, occupies around 220 kb of the human chromosome 6p21, and is organised genomically similarly to the other genes. (2,3) Two promoters that are separated by a lengthy intron and exon 1 are in charge of regulating the expression of the runx2 gene. Two main transcripts, type II (which begins with the sequence MASNS) and type I (which begins with the sequence MRIPV), are produced by the distal and proximal promoters (P1 and P2, respectively). Although both protein isoforms are expressed by osteoblasts and terminal hypertrophic chondrocytes, numerous additional non-osseous tissues have also been shown to express the type I isoform. It has also been claimed that Runx2 has a third isoform; however, human beings do not possess this isoform. Last but not least, alternative splicing has been shown to produce extra Runx2 isoforms, each of which has been proposed to perform a different cellular role. (4)

The cells called osteoblasts are responsible for maintaining and repairing bone, and Runx2 is essential for osteoblast differentiation, function, and ultimately bone biology. Runx2 determines the osteoblast lineage from multipotent mesenchymal stem cells in the early stages of embryogenesis, but suppresses it in the late stages. Runx2 is first identified during development on day 9.5 in the notochord and is substantially expressed in all growing skeletal parts by day 10.5 of the embryo.(5) All of the main osteoblast-related genes' promoter regions contain OSE2, which regulates the expression of all of these genes and allows Runx2 to function. Genes that are exclusive to osteoblasts, such as osteocalcin, alkaline phosphatase, collagenase-3 (matrix metalloproteinase-13, MMP-13), bone sialoprotein, and collagen type Ialpha1, are up-regulated as a result of Runx2's ectopic expression in mesenchymal cell lines. (6)

Runx2 is necessary for the development of bone, but transgenic mice that overexpress it specifically in certain tissues develop osteopenia, a decrease in bone mineral density, and numerous fractures. These results have shown that Runx2 expression and activity are tightly regulated and important for bone (patho)biology. (7)

Results from several studies suggest that type I and type II of the two main Runx2 isoforms (type I and type II) support distinct functions at different stages of osteoblast differentiation and in the two processes of bone formation, namely endochondral and intramebranous bone formation. Both isoforms are involved in the stimulatory action of osteoblast differentiation. To this point, type I isoform appears to play a major role in the creation of intramedullary bone, whereas type II isoform only plays a function in the formation of endochondral bone. (8)

MiRNAs are now regarded as one of the most effective regulators at the post-transcriptional level of gene expression, especially in light of the emergence of epigenesis and related regulatory mechanisms. (9) Non-coding RNAs called miRNAs are 20–22 nucleotides long, and they control protein synthesis by silencing genes at the post-transcriptional level by interacting with mRNA at the 3′UTR (untranslated region). Complementarity between miRNA and mRNA regulates the gene by either preventing translation or cleaving the mRNA, which is made possible by the binding of the miRNA's 5′ end to the mRNA's 3′ UTR. Although some miRNA gene loci also originate in the exonic sections of structural genes, intronic regions of non-coding and coding genes are where miRNA gene loci are most frequently found. (10) The precursor transcript for mature miRNA is created in the cytoplasm by the cleavage of a hairpin loop after processing of a primary transcript (about 80 nt) by the RNase III enzyme Drosha. Later, to target and control the relevant mRNA, one strand from the miRNA duplex is integrated into the RNA-induced silencing complex (RISC). Numerous biological functions, including cell division, proliferation, differentiation, and survival, are regulated by miRNAs. MiRNAs are essential for the post-transcriptional control of genes involved in differentiation in osteoblasts. By specifically targeting Runx2, for instance, miR-204 and miR-133 act as negative regulators of osteogenesis. Runx2 was the target of miR-433, which prevented osteoblast development in BMP-2-induced mouse MSCs (mMSCs, C3H10T1/2).(11)

Many life-saving medications, cosmetics, and dietary supplements have been made primarily from natural ingredients, primarily those derived from plants. The majority of pharmaceuticals and healthcare items, including semi-synthetic medications, that are sold today are either directly derived from or inspired by natural products. Thousands of natural compounds have already been tested for their biological effectiveness against a range of human diseases, and a large number of them were discovered to be potentially active. (12)However, most of these molecules, including emodin, quercetin, myricetin, kaempferol, sitosterol, rutin, and luteolin, could not be developed as an essential drug in their pure form, despite being used in the form of a crude extract or herbal formulation. This was due to limitations of stability, solubility, bioavailability, or toxicity. In addition, difficulties with their isolation, identification, accessibility, and cost are the additional problems causing pharmaceutical businesses to be less interested in using them for generating new drugs. Interestingly, the scientific community is still researching natural remedies due to their therapeutic potential, particularly against chronic diseases and infections brought on by MDR pathogens. These diseases include cancer, diabetes, hypertension, and rheumatoid arthritis, all of which are currently treatable but incurable.(13)

For the in vitro modulation of cell biological characteristics, bioflavonoids are projected to replace growth factors due to their wide range of biological activities. A naturally occurring bioflavonoid called rutin is extensively distributed in plants. Rutin can be utilised to treat tumours, inflammation, and tumor-related conditions by having antioxidant and anti-free radical actions. Rutin is a typical bioflavonoid that is affordable, secure, and simple to get. One of the frequent secondary metabolites of plants, rutin is a naturally occurring flavonoid glycoside also known as rutoside, quercetin-3-O-rutinoside, sophorin, or vitamin P. Chemically, it is referred to as 2-(3,4-dihydroxyphenyl).-5,7-dihydroxy-3-[α-l-rhamnopyranosyl-(1→6)-β-d-glucopyranosyloxy]-4H-chromen-4-one. It has a molar mass of 610.521 g/mol and is a yellowish powder. It rapidly dissolves in pyridine but is poorly soluble in water. Rutin has a pKa value of 6.17 and melts at 125 °C. Rutin can stimulate cell proliferation and osteogenic differentiation in vitro, which can effectively prevent and treat osteoporosis, according to research. The present study put forth the hypothesis to find out the role of Rutin on regulation of miRNA targeting Runx2. (14)

MATERIALS AND METHODS:

Place of study: Department of Forensic Odontology, Saveetha Dental College and Hospitals, Chennai, Tamilnadu, 600077.

National Centre for Cell Science in Pune, India provided the MC3T3-E1 cells, which are the precursors of Human osteoblast cells . At 37°C and 5% CO2, routine culture was carried out using the -MEM medium containing 10% FBS and 1% P/S. MC3T3-E1 cells' status was changed. Randomly selected MC3T3-E1 cells were separated into two groups: a control group and a rutin-1 group (0.01 mmol/L).

The Rutin group received the highest dose of the drug solvent; two to three days later, the solvent was replaced with new osteogenic induction fluid containing rutin. The Control group received no treatment. The cells were harvested for further tests after a predetermined amount of regular culture time. Sigma Pharmaceuticals sold the drug rutin.

Step 1: The list of miRNAs targeting Runx2 is predicted using miRDB database (online tool)

Step 2: Top 10 miRNAs alone is represented in the table and top most miRNA is selected

Step 3: The potential positions where the miRNA binds to Runx2 (seed region) is identified using Targetscan data base (online tool)

Step 4: Human osteoblast cells were treated with Rutin (0.1 mM) for 3days under normal medium

Step 5: hsa-miR-889-5p expression was studied in these cells by

realtime PCR analysis (under rutin treatment)

Real time PCR:
After three days of rutin treatment, cells from all groups were collected. The TRIpure extraction kit (RP1001; BioTek, Beijing, China) instructions were followed to extract the total RNA from cell samples from each group. The amount of RNA in each sample was measured using the UV spectrophotometer NanoDrop-2000 (ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription was carried out using super M-MLV reverse transcriptase (PR6502; BioTeke, Beijing, China) and real-time PCR (Exicycler 96, BIONEER, Republic of Korea). The 2Power Taq PCR MasterMix (PR1702; BioTeke), SYBR Green I (SY1020; Solarbio), cDNA template, and upstream and downstream primers were added to the real-time fluorescence quantitative PCR experiment. B-actin served as a guide when the reaction was finished. The equation 2Ct was utilized to calculate the relative expression level of mRNA of Runx2 gene.
Western blot:
Rutin was applied to the cells in one group for three days. After being divided in half using RIPA lysis buffer (P0100; Solarbio), the treated cells were centrifuged at 12,000 rpm and 4°C for ten minutes. Protein extract was created by separating the supernatant. To determine the protein content, the total protein (TP) extracted was subjected to a quantitative analysis using a BCA protein assay kit (WLA004, Wanleibio, Shenyang, China). The preparation of polyacrylamide gel and assembly of the electrophoresis device. Electrophoresis was performed using 8% SDS-PAGE. The distinct protein bands were then transferred to the PVDF membrane following electrophoresis.
5% skim milk was used to seal the PVDF membrane before the addition of the 1:500-diluted Runx2 primary antibody (WL03358; Wanleibio). Overnight, the PVDF membrane was incubated at 4°C. After the incubation period was up, TBST was used to wash the PVDF membrane. For incubation, horseradish peroxidase (HRP)-labeled sheep anti-rabbit IgG (SE134; Solarbio, diluted 1:5000) was applied. The same experimental techniques were used to incubate the reference -actin. Then, for exposure in a darkroom, ECL luminous fluid (PE0010; Solarbio) was added. A scan was done on the film. The optical density of the target band was analyzed using the gel image processing system (the program Gel-Pro-Analyzer).
Statistical analysis:
SPSS 20.0 was used to analyze the data. Means SD were used to express all results. The chi square test was used to analyze the counting data; a difference was considered statistically significant if p0.05 (*p0.05, **p0.01, ***p0.001). Charts were created using GraphPad Prism 8.0 program.
Duration of study: 3 months

Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
1	99	hsa-miR-889-5p	RUNX2	runt related transcription factor 2
2	99	hsa-miR-3148	RUNX2	runt related transcription factor 2
3	98	hsa-miR-196a-1-3p	RUNX2	runt related transcription factor 2
4	98	hsa-miR-135b-5p	RUNX2	runt related transcription factor 2
5	98	hsa-miR-135a-5p	RUNX2	runt related transcription factor 2
6	98	hsa-miR-187-5p	RUNX2	runt related transcription factor 2
7	98	hsa-miR-605-3p	RUNX2	runt related transcription factor 2
8	97	hsa-miR-5088-3p	RUNX2	runt related transcription factor 2
9	96	hsa-miR-30a-5p	RUNX2	runt related transcription factor 2
10	96	hsa-miR-30c-5p	RUNX2	runt related transcription factor 2

Figure 1: Predicted miRNAs targeting Runx2. Data was retrieved from MirDB database

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}	Predicted relative K_D
Position 161-168 of RUNX2 3' UTR hsa-miR-889-5p	5' ...UCAGUCAUGAUCUUGCAGCCAUA... 3' CUGGUAUGAUGCCUGUCGGUAA	8mer	-0.23	91	-0.23	0.031	N/A	N/A
Position 774-780 of RUNX2 3' UTR hsa-miR-889-5p	5' ...GCAAUACAUUAUUAUAGCCAUAA... 3' CUGGUAUGAUGCCUG-UCGGUAA	7mer-A1	-0.16	82	-0.16	0.031	N/A	N/A
Position 1015-1021 of RUNX2 3' UTR hsa-miR-889-5p	5' ...UCUUCAGUGGUCUAGAGCCAUAU... 3' CUGGUAUGAUGCCUGUCGGUAA	7mer-A1	-0.09	69	-0.09	0.031	N/A	N/A

Figure 2: Potential target regions in 3'-UTR of Runx2 gene by hsa-miR-889-5p

RESULTS:

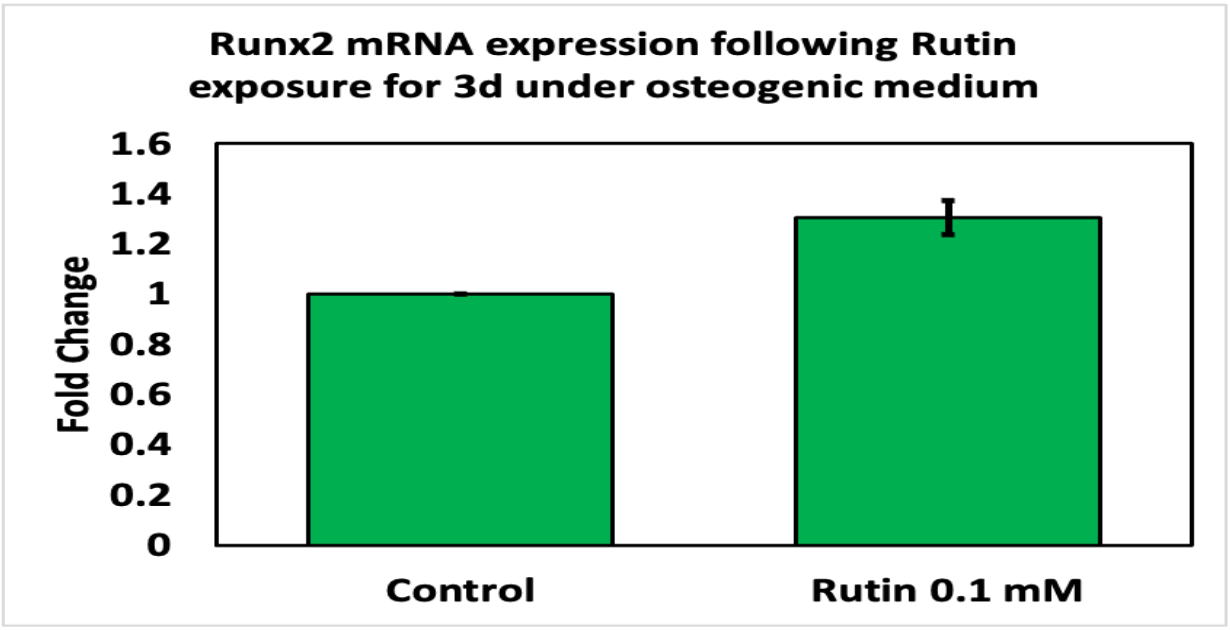

Figure 3: From the above graph we can infer that the expression of Runx2 mRNA has been increased after the exposure of Rutin for 3 days under osteogenic medium.

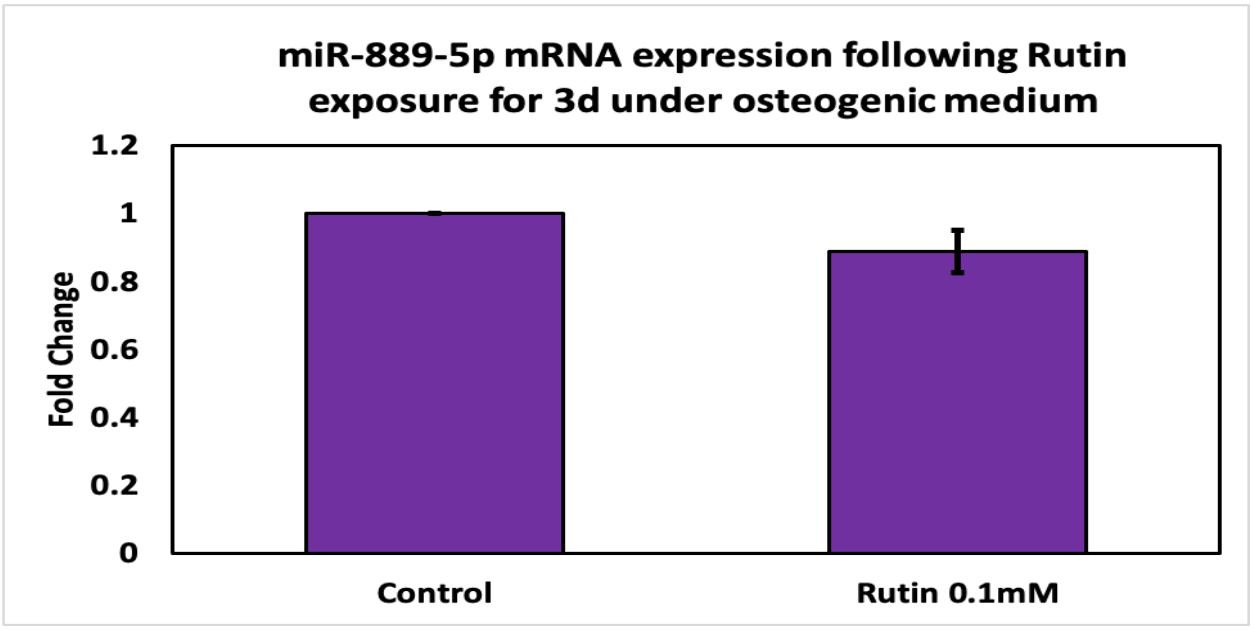

Figure 4: From the above graph we can infer that the expression of miR-889-5p mRNA has been reduced after the exposure of Rutin for 3 days under osteogenic medium.

From the above results, it can be concluded that Rutin upregulates the expression of Runx2 and suppress the expression of miR-889-5p mRNA, there by promoting osteoblast differentiation and the related genes to be expressed in the osteoblast.

Rutin treatment for 3 days caused the expression of the Runx2 gene to be discovered by PCR in MC3T3-E1 cells. The findings demonstrated that concentrations of rutin could greatly enhance the quantity of Runx2 gene expression when compared to the untreated control group, and there were significant differences (p0.05).

The expression of the Runx2 protein was found by Western blot and IHC after the group's cells were exposed to rutin for three days. When compared to the untreated control group, the WB results demonstrated that rutin can dramatically boost the expression level of the Runx2 protein. Additionally, the Runx2 protein's expression level increased, and there were noticeable differences (p 0.05). Increasing the comparison with rutin concentrations may produce consistent, significant data that can be determined by WB.

DISCUSSION:

Rutin is a flavonoid that is present in a variety of plants and has a wide range of biological functions, including the ability to fight off oxidation and inflammation, safeguard the liver and nerves, and stop brain hemorrhage. The significance of rutin in

additional disorders has progressively come to light, in addition to those that are known to exist and have been clinically treated with it. Rutin has significant potential for both the prevention and treatment of diabetes, according to earlier studies. (15)People have also gradually come to understand how rutin affects orthopedic problems. Osteoporosis is a chronic illness whose primary cause is the loss of the mechanical flexibility and mineralization capacity of the bone.(16)

Considering that the currently prescribed anti-osteoporosis medications have some effectiveness but also some negative side effects, such as a higher risk of blood clots and cancer, phytochemicals might be a better and safer alternative. Rutin can boost the expression of genes associated to osteocytes and osteoblasts while decreasing the expression of genes related to osteoclasts and the Runx inhibitor, according to a study on human osteosarcoma cells called SAOS-2 cells. The research on rutin's effects on ALP activity showed that rutin can raise ALP activity while lowering acid phosphatase activity, which is a sign of osteoporosis. Rutin can therefore encourage osteocyte proliferation and serve as a marker for ossification.(17)

The findings of this study are in line with those of our investigation, demonstrating that rutin is a potent medication that can be utilized to treat orthopedic illnesses in the future. Xiao et al. investigated the mechanism of action of rutin in osteoporosis and discovered through analysis using a variety of experimental techniques, including Micro-CT, Western-Blotting, Real-time PCR, transmission electron microscope, and alizarin red staining, that rutin can regulate FNCD1 level and autophagy through the Akt/mTOR signaling pathway, providing a new approach for the treatment of osteoporosis. (18)Rutin is expected to be a new potential medicine for the treatment of periodontitis bone abnormalities because it can shield human periodontal ligament stem cells (HPDLSCs) from the TNF-induced osteogenic differentiation damage in an inflammatory environment.

Osteoblasts create new bone while osteoclasts break down existing bone to maintain a dynamic equilibrium in the development and regeneration of bone. One of the crucial members of the Runt family is Runx2, often referred to as core-binding factor al. Runt1 and Runx3 make up the other two individuals. According to certain research, the Runx2 gene can cause the creation of bone matrix protein at an early stage of osteoblast development and produce a large number of immature osteoblasts.(19) As a particular marker gene, it can be utilized to show osteoblast differentiation and bone creation. Additionally, via binding to cis-acting components of osteoblasts, the Runx2 gene stimulates the transcription and production of the bone sialoprotein, osteopontin, and type I collagen genes. Osteoprotegerin (OPG), a molecular substance made by osteoblasts, is crucial for the development of osteoclasts because it has the ability to both absorb bone and prevent the growth of osteoclast-like cells in vivo and in vitro. Human OPG sequence cloning revealed a binding site for OPG in the Runx2 gene sequence, demonstrating Runx2's ability to control OPG expression. One of the main factors influencing the development of multiple myeloma is the runx2 gene, which is abundantly expressed in these cells. Multiple myeloma can be slowed down by controlling the Runx2 upstream genes to reduce the amount of Runx2. The outcomes match those of our study. The Runx2 gene will undoubtedly give a detailed theoretical foundation and practical value for the treatment of bone-related illnesses with further research and knowledge of its more complex activities and mechanism of action.

CONCLUSION:

Rutin was able to promote osteoblastic differentiation by upregulating Runx2 and by directly reducing the level of microRNA 889 5p. Rutin therapy for three days led to the discovery by PCR of Runx2 gene expression in MC3T3-E1 cells. When compared to the untreated control group, the results showed that concentrations of rutin may significantly increase the quantity of Runx2 gene expression (p0.05).

AUTHORS CONTRIBUTION:

All authors have equally contributed.

NAME OF THE FUNDING AGENCY:

The present project is funded by
Saveetha Institute of Medical and Technical Sciences
Saveetha Dental College and Hospitals
Saveetha University
Kumaran Department Stores

ACKNOWLEDGMENT:

We extend our sincere gratitude to Saveetha Dental College and hospitals for their constant support and successful completion of this work.

CONFLICT OF INTEREST:

The authors hereby declare that there is no conflict of interest in this study.

ETHICAL CLEARANCE: Since it is an in vitro study ethical clearance is not needed.

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