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STUDYING THE LANDSCAPE OF SMAD7 TARGETING MIRNAS REGULATED BY CALCIUM SILICATE PARTICLES

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Abstract
Aim:- To identify the miRNAs which target Smad7 protein, and identify the mechanism of regulation of Smad7 targeting miRNAs by calcium silicate particles. **Introduction:-** Smad proteins are negative regulators of the bone morphogenic protein (BMP) and the transforming growth factor-β (TGF-β) signaling pathways. They are regulated by miRNAs which either promote or suppress their expression. Calcium silicate (CS) is an amorphous crystal used as a drug carrier and promoter in osteogeneration. **Materials & Methods:-** The list of miRNAs targeting Smad7 is predicted using an online miRNA database called miRDB. The potential positions or seed regions where the miRNA binds to Smad7 is identified using an online database TargetScan. Human osteoblast cells are treated with a conditioned medium which contains calcium silicate. The expression of Smad7 and hsa-miR-4524a-5p are studied in the osteoblast cells by real time polymerase chain reaction (real time PCR). **Results:-** The main miRNAs that target Smad7 — hsa-miR-4524a-5p and hsa-miR-4524b-5p — are upregulated by calcium silicate, which in turn leads to the downregulation of Smad7. This inhibits the expression of the TGF-beta-BMP pathway which plays a vital role in osteoclastic activity and bone remodeling. **Discussion:-** Smad7 stimulates the binding of Smad2 and Smad3 to Smad4 for their expression by the TGF-β-BMP pathway. The main miRNAs that target Smad7 are hsa-miR-4524a-5p and hsa-miR-4524b-5p. These miRNA are upregulated by calcium silicate, which in turn leads to the downregulation of Smad7. This inhibits the expression of the TGF-β-BMP pathway which plays a vital role in osteoclastic activity and bone remodeling. Thus bone formation and bone remodeling is enhanced. **Conclusion:-** By understanding the molecular mechanism of miRNA regulation, we have evaluated the osteogenic potential of calcium silicate. The significant elevation in hsa-miR-4524a-5p expression leading to the downregulation of Smad7 expression is responsible for the increased osteogenesis.
KEYWORDS: calcium silicate, Smad7, miRNA, mRNA, microRNA, osteogenesis, bone morphogenic protein, BMP, bone remodeling

INTRODUCTION

RNAs or ribonucleic acids are single stranded polymeric molecules with alternating phosphate and ribose groups. They are essential in various biological roles in coding, decoding, regulation and expression of genes. Among the functional RNA molecules, non coding RNAs are not translated into proteins. Such RNAs include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), small nuclear RNAs (snRNAs) and so on. (Macfarlane and Murphy 2010)

MicroRNAs (miRNAs) are a small group of non coding RNAs that target messenger RNAs (mRNAs). They were first discovered in *Caenorhabditis elegans.* An miRNA is the result of the transcription of an approximately 60 to 70 nucleotide long RNA which has a stern loop or hairpin loop structure. They control gene expression and regulate a wide array of biological processes by inducing translational repression or RNA

degradation. miRNAs are produced naturally by cells from genome encoded sequences which are made up of their own promoter elements. (Bavelloni et al. 2017)

The aberrant expression of even a single miRNA can alter the translation of several genes within a cell, and subsequently cause mutations in the cell phenotype. miRNA modification can alter their function as well as their target specificity. Altered miRNA expression can affect several aspects of cellular function including, and not limited to, proliferation and differentiation, apoptosis and so on. miRNA deficiencies or excesses have also been associated with a number of clinical conditions ranging from cancer to myocardial infarction. miRNAs interact among themselves and these interactions have been shown to drive oncogenic pathways. (Hill and Tran 2021)A case control study on Alzheimer's disease shows the increasing depression in the expression of miRNA-210 in the cerebrospinal fluid and serum with increasing severity of the Alzheimer's condition. (Zhu et al. 2015)

Osteogenesis is a complex process which involves the differential activities of multiple types of cells for the formation and remodeling of the skeletal framework. miRNAs repress levels of cellular proteins to provide a sophisticated mechanism of gene regulation. Deregulation of miRNA mediated mechanisms has emerged as an important pathological factor in bone resorption or bone degenerative disorders such as osteoporosis. (van Wijnen et al. 2013)The inhibition of translation of mRNA by targeting their specific miRNAs has emerged as an effective regulator of the developmental signaling pathways of osteogenesis, growth and differentiation of osteoblasts, bone resorption mediated by osteoclasts and bone homeostasis in the adult skeleton. miRNAs control multiple levels and types of gene regulation which are essential for bone development and postnatal osteogenic functions, from the initial response of progenitor cells or stem cells to the structural and metabolic activities of the mature osseous tissue. (Lian et al. 2012)

Smad proteins are signal transducers for the members of the transforming growth factor-β (TGF-β) superfamily. Bone morphogenic proteins (BMPs) have a stimulatory effect on the conversion of precursor cells, such as the undifferentiated C2C12 murine mesenchymal cells, into osteoblasts. (Matsuzaki 2013) Smad proteins mediate the signal transduction in the TGF-β-BMP signaling pathway that affects both osteoblast and osteoclast functions, and therefore plays a critical role in bone remodeling. Recent studies demonstrate that targeting the Smad dependent TGF-β-BMP signaling pathway might be a novel and promising therapeutic strategy against osteoporosis. (Zou et al. 2021)

Nanostructured calcium silicate based materials are highly biocompatible and have a highly porous hollow structure. The biodegradability and high drug loading capacity of calcium silicate nanosystems provide promising applications for the same in drug delivery applications. (Zhu et al. 2017) The long drug release time of the system significantly prolongs the therapeutic effect of the drugs delivered. The pH responsive drug delivery exhibited by the calcium silicate nanosystems is an advantage as it facilitates targeted drug delivery. (Huang et al. 2017)

Mesoporous calcium silicate nanoparticles maintain sustained release of gentamicin and fibroblast growth factor FGF-2, which promotes bone matrix formation. Due to the FGF-2 release, the MesoCS nanospheres produce higher stimulation of odontogenic protein. MesoCS extracts also significantly stimulate the expression of osteogenic genes such as OPN, ALP and OCN in periodontal ligament cells. This indicates the excellent osteostimulation property of calcium silicate nanospheres. (Wu et al. 2012)

The aim of this study is to identify the molecular mechanism for the osteogenic role of calcium silicate, identify the miRNAs which target Smad7 protein and to identify the regulation of Smad7 targeting miRNAs by calcium silicate particles.

MATERIALS & METHODS

Study Setting

This *in vitro* study was conducted in a university setting at Saveetha Dental College and Hospital, Chennai, India for a duration of 3 months from April 2023 to June 2023.

Data Collection

The list of miRNAs targeting Smad7 was predicted using an online miRNA database called miRDB. The miRNAs with the highest targeting influence on Smad7 called hsa-miR-4524a-5p and hsa-miR-4524b-5p were identified and selected for further analysis.

The potential positions or seed regions where the miRNAs hsa-miR-4524a-5p and hsa-miR-4524b-5p bind to Smad7 were identified using an online database called TargetScan.

Study Groups

A growth medium was prepared by adding calcium silicate to 100% conditioned culture medium for 3 days. Osteogenic inducers such as ascorbic acid and β-glycerophosphate were added to the culture, and the prepared medium was incubated at 37°C room temperature and 5% CO2.

Human osteoblasts (MG-63 cells) were seeded in the conditioned culture medium in tissue culture plates and allowed to proliferate, to obtain the experiment study group of treated osteoblast cells.

Untreated osteoblast cells were taken as the control group.

Real Time Polymerase Chsin Reaction (Real Time PCR)

- Following calcium silicate treatment, RNA is extracted from the cells using a commercially available RNA extraction kit according to the manufacturer's instructions.
- Specific primers for Smad7 and hsa-miR-4524a-5p were designed and optimized.
- The expression of Smad7 and hsa-miR-4524a-5p were studied in both the study groups of osteoblast cells by real time polymerase chain reaction (real time PCR).
- Real Time PCR reactions were carried out using Smad7, hsa-miR-4524a-5p, gene-specific primers and a suitable master mix. The amplification conditions were optimized for efficient and specific amplification.
- The data obtained was analyzed using appropriate statistical methods (e.g., t-test or ANOVA) to determine significant differences in the gene expression between the two study groups.

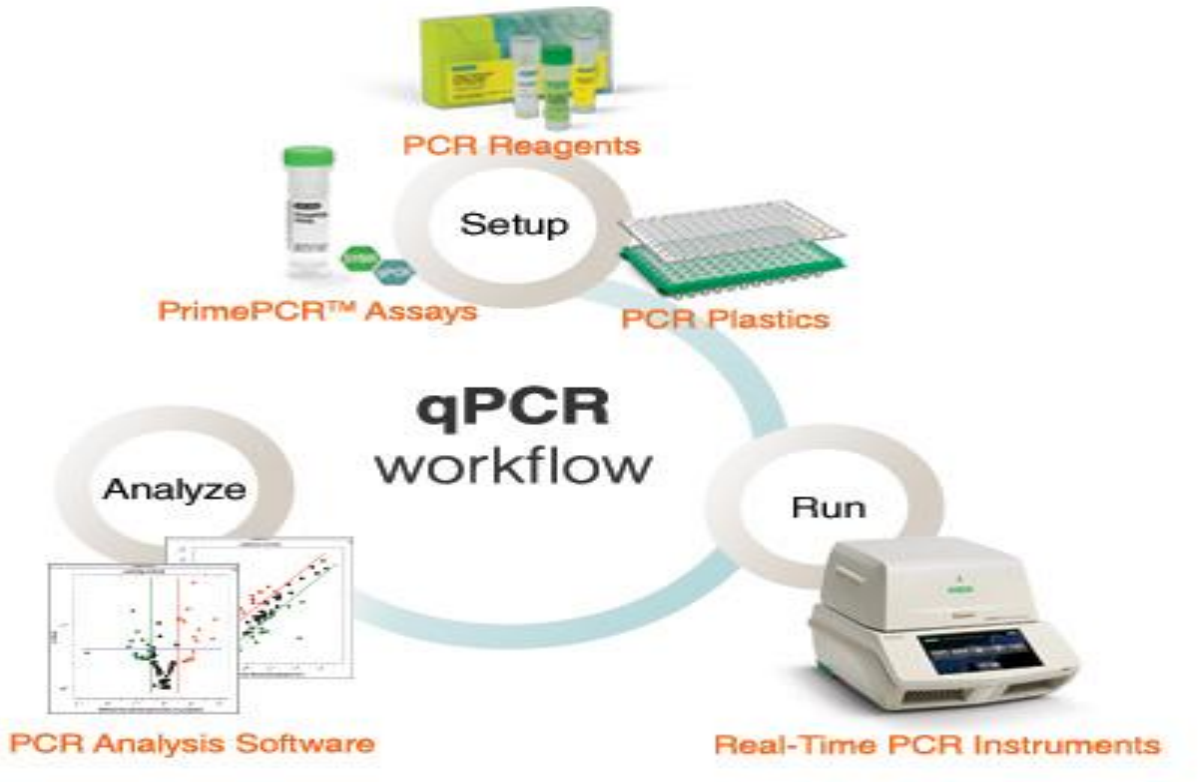

Figure 1:- Diagrammatic representation of real time polymerase chain reaction (real time PCR)

RESULTS

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	97	hsa-miR-4524a-5p	SMAD7	SMAD family member 7
Details	2	97	hsa-miR-4524b-5p	SMAD7	SMAD family member 7
Details	3	96	hsa-miR-410-3p	SMAD7	SMAD family member 7
Details	4	95	hsa-miR-5680	SMAD7	SMAD family member 7
Details	5	95	hsa-miR-3926	SMAD7	SMAD family member 7
Details	6	95	hsa-miR-627-3p	SMAD7	SMAD family member 7
Details	7	94	hsa-miR-5011-5p	SMAD7	SMAD family member 7
Details	8	93	hsa-miR-4803	SMAD7	SMAD family member 7
Details	9	93	hsa-miR-424-3p	SMAD7	SMAD family member 7
Details	10	92	hsa-miR-122b-3p	SMAD7	SMAD family member 7
Details	11	92	hsa-miR-15b-5p	SMAD7	SMAD family member 7
Details	12	92	hsa-miR-16-5p	SMAD7	SMAD family member 7
Details	13	92	hsa-miR-21-3p	SMAD7	SMAD family member 7
Details	14	92	hsa-miR-15a-5p	SMAD7	SMAD family member 7
Details	15	92	hsa-miR-195-5p	SMAD7	SMAD family member 7
Details	16	91	hsa-miR-497-5p	SMAD7	SMAD family member 7
Details	17	91	hsa-miR-6838-5p	SMAD7	SMAD family member 7
Details	18	91	hsa-miR-424-5p	SMAD7	SMAD family member 7
Details	19	90	hsa-miR-766-5p	SMAD7	SMAD family member 7
Details	20	89	hsa-miR-374b-3p	SMAD7	SMAD family member 7

Figure 2:- List of miRNAs predicted to be targeting Smad7

From the above list, we see that the miRNAs which have the highest influence on Smad7 are hsa-miR-4524a-5p and hsa-miR-4524b-5p, with target scores of 97.

	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 56-63 of SMAD7 3' UTR hsa-miR-4524a-5p	5' ...ACUUCAAACUACUUUGCUGCUAA... 3' ACUCUGUCCAAGUACGACGAUA	8mer	-0.53	99	-0.53	0	N/A	-5.601
Position 56-63 of SMAD7 3' UTR hsa-miR-4524b-5p	5' ...ACUUCAAACUACUUUGCUGCUAA... 3' CUCUGUCCGAAUACGACGAUA	8mer	-0.54	99	-0.54	0	N/A	-5.601

Figure 3:- Potential target regions in 3'-UTR of Smad7 gene for miRNAs hsa-miR-4524a-5p and hsa-miR-4524b-5p

From the above data, we can see that the target region for pairing is the 8mer site CGACGAU in case of both hsa-miR-4524a-5p and hsa-miR-4524b-5p with the 3'UTR site GCUGCUA of Smad7.

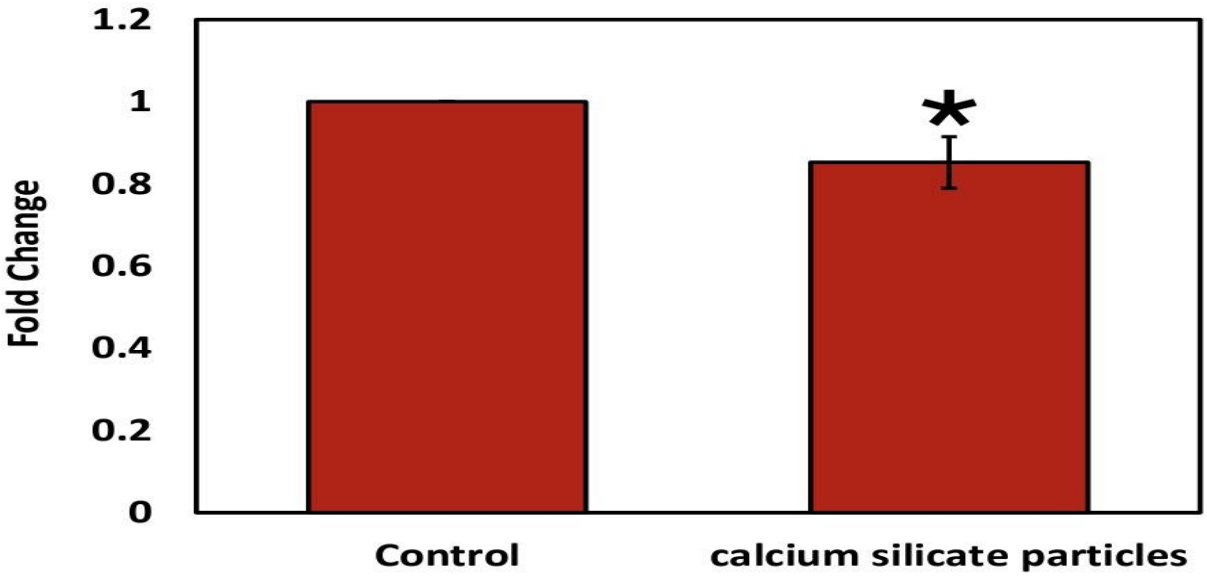

Figure 4:- Expression pattern of Smad7 in human osteoblasts (MG-63 cells) following calcium silicate particle exposure under osteogenic supplementation

From the above graph we can visualize that by the addition of calcium silicate particles there is a decrease in the fold change of Smad7, which shows that the presence of calcium silicate decreases the gene expression of Smad7.

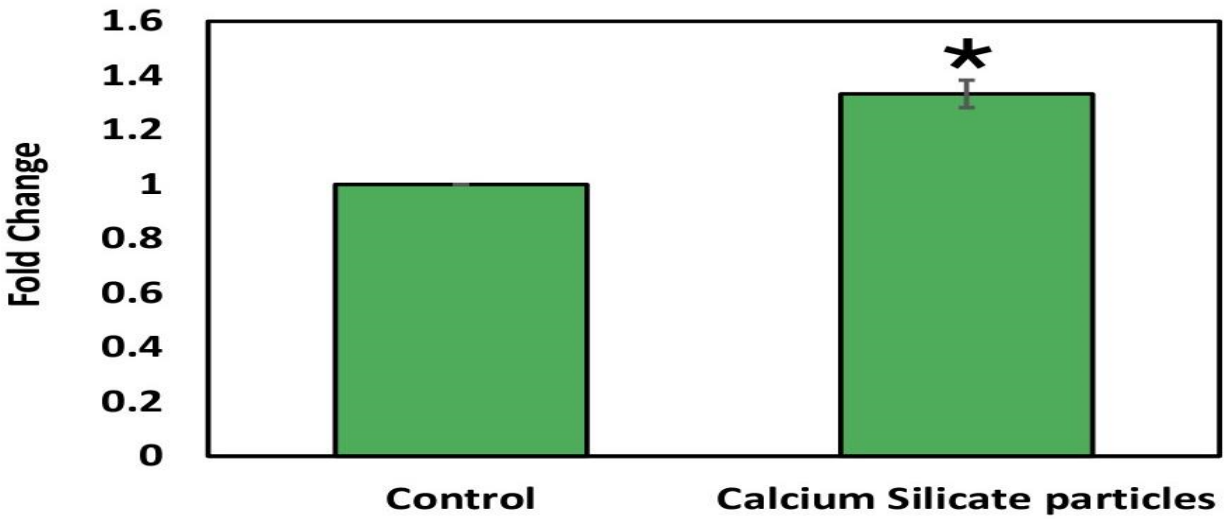

Figure 5:- Expression pattern of hsa-miR-4524a-5p in human osteoblasts (MG-63 cells) following calcium silicate particle exposure under osteogenic supplementation

From the above graph we can visualize that by the addition of calcium silicate particles there is an increase in the fold change of hsa-miR-4524a-5p, which shows that the presence of calcium silicate increases the miRNA expression of hsa-miR-4524a-5p.

DISCUSSION

The aim of this study was to identify the mechanism of action of calcium silicate in osteogenesis. From previous literature we see that the main pathway for osteogenesis is the transforming growth factor beta (TGF-β) and bone morphogenic protein (BMP) pathway (TGF-β-BMP). This pathway is primarily influenced by Smad proteins, since they are signal transducers for the TGF-β superfamily.

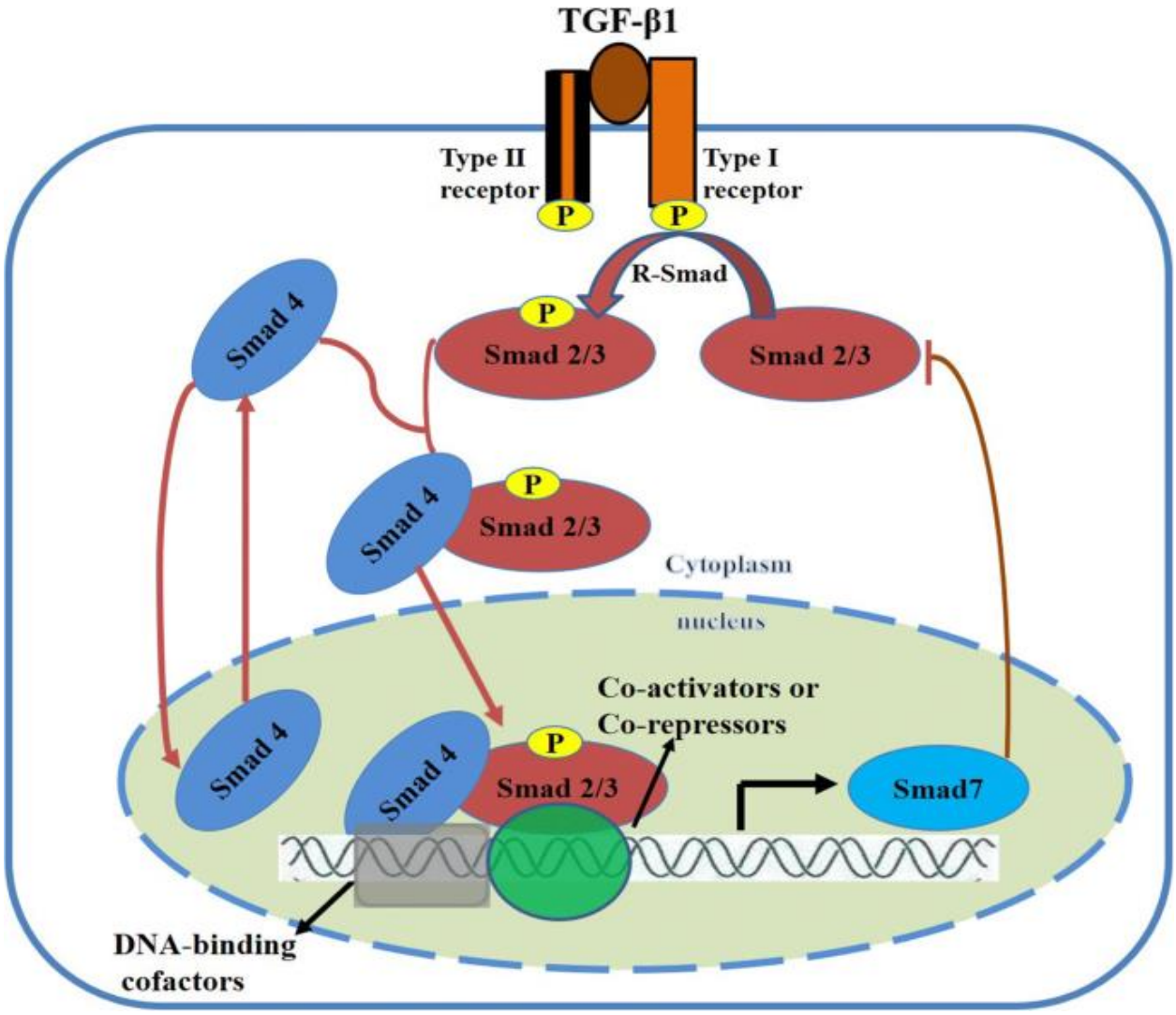

Figure 6:- Diagrammatic representation of the TGF-β-BMP pathway showing the mechanism of action of Smad proteins

The Smad7 protein stimulates the binding of Smad2 and Smad3 to Smad4, which leads to the expression of the TGF-β-BMP pathway. The expression of the pathway leads to the stimulation of osteoclast activity. From Figure 2, we see that the miRNAs that target Smad7 are primarily hsa-miR-4524a-5p and hsa-miR-4524b-5p. Regulation of the activity and expression of these miRNAs subsequently affect the expression of Smad7, affecting the pathway.

Calcium silicate particles have been researched and analyzed as a drug delivery system for osteostimulative drugs and proteins due to its promoting activity on bone formation. The addition of CS particles into the osteoblast conditioning medium enhances their potential for bone formation and bone remodelling. The mechanism of action of this potentiation has been analysed in our present study.

The real time polymerase chain reaction (real time PCR) tests show the levels of expression of the Smad7 gene as well as the influencing miRNAs in the osteoblastic TGF-β-BMP pathway, and the differences in the same when the osteoblast cells are treated with calcium silicate.

From Figure 4, we see that the presence of calcium silicate decreases the gene expression of Smad7. From Figure 5, we see that the presence of calcium silicate increases the miRNA expression of hsa-miR-4524a-5p. Knowing that the miRNAs hsa-miR-4524a-5p and hsa-miR-4524b-5p have influence on Smad7, from both of the above results, we can conclude that the increased expression of the miRNAs by the influence of calcium silicate subsequently decreases the expression of Smad7 in the TGF-β-BMP pathway. This reduced expression of the osteoclastic pathway leads to decreased bone resorption and increased bone formation.

This mechanism of action enables us to use more molecular drugs for the treatment of osteogenic disorders such as osteoporosis. The development of gene repressor or miRNA

enhancer drugs which target the TGF-β-BMP pathway infiltrated with calcium silicate can promote effective regulation of the osteoclastic pathway and prevent bone resorption, while at the same time promoting osteogenic remodeling bone formation.

Previous literature shows the work done on the effect of Smad6 and Smad7 on BMP mediated growth arrest and apoptosis (Ishisaki et al. 1999) and the authors reported that the ectopic expression of Smad7 blocks activity induced cell cycle arrest and apoptosis. Smad7 was confirmed to bind to miRNA-21 and thus downregulated in osteoporotic individuals. (Jiang et al. 2018)

CONCLUSION

By understanding the molecular mechanism of miRNA regulation, we have evaluated the osteogenic potential of calcium silicate. The significant elevation in hsa-miR-4524a-5p expression leading to the downregulation of Smad7 expression is responsible for the increased osteogenesis. This is a preliminary study where we have analyzed the molecular mechanism of action of calcium silicate in osteogenesis. Thus this study opens up many avenues for the employment of calcium silicate and similar compounds for biomedical applications in the treatment of bone disorders or diseases.

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   Study design, data verification, manuscript drafting

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to disclose.

ETHICAL APPROVAL

As this is an in vitro study, the need for ethical approval is void.

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