Syringic acid, a phenolic acid, promotes osteoblast differentiation by stimulation of Runx2 expression and targeting of Smad7 by miR-21 in mouse mesenchymal stem cells

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Abstract

Syringic acid (SA), a phenolic acid, has been used in Chinese and Indian medicine for treating diabetes but its role in osteogenesis has not yet been investigated. In the present study, at the molecular and cellular levels, we evaluated the effects of SA on osteoblast differentiation. At the cellular level, there was increased alkaline phosphatase (ALP) activity and calcium deposition by SA treatment in mouse mesenchymal stem cells (mMSCs). At the molecular level, SA treatment of these cells stimulated expression of Runx2, a bone transcription factor, and of osteoblast differentiation marker genes such as *ALP*, type I collagen, and osteocalcin. It is known that Smad7 is an antagonist of TGF- β /Smad signaling and is a negative regulator of Runx2. microRNAs (miRNAs) play a key role in the regulation of osteogenesis genes at the post-transcriptional level and studies have reported that *Smad7* is one of the target genes of miR-21. We found that there was down regulation of *Smad7* and up regulation of miR-21 in SA-treated mMSCs. We further identified that the 3'-untranslated region (UTR) of *Smad7* was directly targeted by miR-21 in these cells. Thus, our results suggested that SA promotes osteoblast differentiation via increased expression of Runx2 by miR-21-mediated down regulation of *Smad7*. Hence, SA may have potential in orthopedic applications.

Keywords Bone · miR-21 · Runx2 · Smad7 · Syringic acid

Abbreviations

ALP	Alkaline phosphatase
COL-I	Type I Collagen-I
DMEM	Dulbecco's modified eagle's medium
HDAC4	Histone deacetylases
MMPs	Matrix metalloproteinases
mMSC	mouse Mesenchymal Stem Cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OC	Osteocalcin
PBS	Phosphate-buffered saline
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SA	Syringic acid

TGF- β	Transforming growth factor- β
TRAP	Tartrate-resistant acid phosphatase

Introduction

Bone is a dynamic tissue which undergoes formation and resorption, simultaneously. It carries out a number of important functions in the body such as support, locomotion, calcium and phosphate storage, and protection of vital organs. Bone tissue is made up of four cell types, namely, osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts make up 4–6% of bone cells and are known as bone forming cells. These cells are responsible for secreting osteoid, which results in the formation of new bone. Osteocytes are the most abundant cells found in bone tissue comprising 90–95% of the total tissue. These cells are responsible for regulating osteoblast and osteoclast function, maintaining calcium and phosphate ion homeostasis, and acting as sensory cells which regulate the loading and unloading of bone in response to stimuli (Stevens 2008; Raggatt and Partridge 2010; Boonrungsiman et al. 2012; Florencio-Silva et al. 2015; Nakashima 2016;

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Katsimbri 2017; Chen et al. 2017a, b; Vishal et al. 2017a; Ono and Takayanagi 2017; Boudin and Van Hul 2017).

Naturally-occurring phytochemicals have several beneficial effects on human health. Plants are the major source of flavonoids and polyphenols (Pandey and Rizvi 2009). Almost 8000 polyphenols have been reported and, of these, only 500 have been shown to be bio-active (Dudaric et al. 2015). Polyphenols help in protection of the bone by upregulation of osteoblastogenesis and downregulation of osteoclastogenesis via several mechanisms involving the action of antioxidants and osteo-immunological activity (Santangelo et al. 2007). One of the functions of polyphenols is to scavenge reactive oxygen species (ROS) and to promote osteoblast differentiation by increasing levels of Runx2, osteocalcin (OC), Wnt, and IGF-1, and also by suppressing RANKL, TRAP, and MMPs expression (Santangelo et al. 2007; Zhou et al. 2015). Defects in Runx2 cause various skeletal disorders including osteoporosis. Runx2 is one of the predominant factors in bone which is required for osteoblast differentiation (Harada et al. 1999; Komori 2010; Muthusami et al. 2011; Moorthi et al. 2013; Vimalraj et al. 2014, 2015; Vishal et al. 2017b; Selvamurugan et al. 2017; Choi et al. 2017; Ling et al. 2017).

MicroRNAs (miRNAs) are small non-coding RNAs, 22–25 nucleotides in length, that regulate gene expression at the post-transcriptional level (Vimalraj and Selvamurugan 2012; Moorthi et al. 2013; Vimalraj et al. 2014; Vimalraj and Selvamurugan 2014; Sainitya et al. 2015; Fang et al. 2015; Selvamurugan et al. 2017; Vishal et al. 2017b; Sera and zur Nieden 2017; Luo et al. 2017). A small number of studies have shown that miRNA expression can be regulated by different polyphenols (Milenkovic et al. 2012; Parasramka et al. 2012; Baselga-Escudero et al. 2014; Zhou and Lin 2014). In this study, we selected syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid; SA), a phenolic acid found in olives, walnuts, and dates. It exhibits several positive effects on human health. SA has strong antioxidant, antihypertensive, anti-proliferative, anti-endotoxic, anti-cancer, hepatoprotective, and anti-hyperglycemic activities. While previous studies have established the antidiabetic properties of SA, to our knowledge, no studies have been conducted regarding its effects on osteogenesis. Hence, the aim of this study was to determine the osteogenic properties of SA and the molecular mechanisms responsible for SA-mediated osteoblast differentiation in mouse mesenchymal stem cells (mMSCs).

Materials and methods

Materials

Cell culture reagents such as DMEM, phosphate buffered saline (PBS), and trypsin were purchased from Lonza, Basel, Switzerland. The antibodies for Runx2, Smad7, HDAC4 and

α -tubulin were obtained from Santa Cruz Biotechnology, Dallas, Texas, USA. Syringic acid and other chemicals for staining were obtained from Sigma (St. Louis, MI, USA). A cDNA synthesis kit was sourced from BioRad, Hercules, CA, USA. SYBR green was obtained from Clontech, Mountain view, California, USA. The primers for Runx2 and RPL13A were synthesized by Sigma, St. Louis, Missouri, USA. mMSCs (C3H10T1/2) were obtained from (NCBS, Pune, India). The cells were maintained in 10% fetal bovine serum (FBS) containing DMEM medium at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

MTT assay

A direct MTT assay was carried out to test the cytotoxicity of SA as described previously (Dhivya et al. 2015; Leena et al. 2017). mMSCs were cultured in DMEM for 2 d. The media was discarded and 200 μ l of 0.05% MTT solution was added to each well. The cells were then incubated for 1 h at 37 °C. DMSO was added to solubilize the formazan crystals and the OD was measured using a spectrophotometer at 570 nm.

ALP staining

ALP activity of the differentiated mMSCs was evaluated as described previously (Vimalraj et al. 2014). The cells were then cultured for 7 d. Upon the completion of the incubation period, the cell-seeded wells were washed with 1 $\times$ PBS then fixed with 10% formalin and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT; Sigma-Aldrich, St. Louis, Missouri, USA) was added and incubated for 2 h. Stained cells were identified under inverted light microscopy as blue in color and were quantified at 620 nm by dissolving the stains using KOH:DMSO.

Alizarin red staining

Analysis of calcium deposits caused by differentiated and mineralized osteoblasts was performed with alizarin red staining, as described previously (Sainitya et al. 2015; Vimalraj et al. 2016; Leena et al. 2017; Saravanan et al. 2017). mMSCs were cultured for 14 d with change of fresh medium once every three days. The cells were then washed with 1 $\times$ PBS and fixed with 10% formalin for 30 min, then rehydrated with 1 mL of distilled water for 5 min. The fixed monolayer of cells was then incubated with 1% alizarin red (pH 4.2) in 2% ethanol for 10 min at room temperature. 1 mL of 70% ethanol was added and allowed to stand for 2 min. The calcified nodules stained red in colour, and micrographs were obtained by inverted light microscopy. The stained cells were also quantified at 405 nm by using 1 N acetic acid at 80 °C and neutralized with liquid ammonia.

Von Kossa staining

Analysis of calcium deposits caused by differentiated and mineralized osteoblasts was conducted by von Kossa staining, as described previously (Sowjanya et al. 2013; Kumar et al. 2014; Saravanan et al. 2015). mMSC were cultured in 6-well plates for 14 d with replenishment of fresh medium once every three days. At the end of the incubation period, cells were washed twice with $1 \times$ PBS, fixed in 10% formalin for 10 min, and washed once with water. Water was removed, 1% silver nitrate solution was added, and the plate was exposed to UV light for 20 min and then rinsed with water. Sodium thiosulfate (5%) was added and after 5 min, the plate was rinsed in water. Calcium phosphate deposits were found as black colored spots. Image J software was used to quantify calcium phosphate deposits, and the percentage of the mineralization area was plotted.

Reverse transcriptase real-time PCR

mMSCs were cultured in 6-well plates and treated with SA at different concentrations (100, 150, and 300 μ M). Total RNA was isolated using a kit from Sigma using the TRIZOL method. Reverse transcriptase was carried out using reverse transcription reagents and cDNA was prepared as described previously (Arumugam et al. 2017). qPCR was performed using Quant Studio 3 Real-Time PCR System (Life technologies, Carlsbad, California, United States) which allowed real-time quantitative detection of the PCR products by measuring the increase in SYBR green fluorescence. This was due to binding of SYBR green to double-stranded DNA. The Ct value was calculated. The relative expression of *Runx2*, *ALP*, *Col1* and *OC* was then calculated after normalization with a house keeping gene, *RPL13*. The primers used for real-time PCR in the study are given in Tables 1 and 2.

Table 1 Primers used for real time PCR in this study

S.No.	Gene	Sequence (5'-3')
1	Runx2	Forward -CGGTCTCCTTCCAGGATGGT Reverse -GCTTCCGTCAGCGTCAACA
2	RPL13AB	Forward -CCTGTTTCCGTAGCCTCATG Reverse -AAGTACCAGGCAGTGACAG
3	ALP	Forward -TTGTGCCAGAGAAAGAGAGAGA Reverse -GTTTCAGGGCATTTCCTCAAGGT
4	OC	Forward -ATGGCTTGAAGACCGCCTAC Reverse -AGGGCAGAGAGAGAGGACAG
5	Col-I A1	Forward -TAACCCCTCCCCAGCCACAAA Reverse -TTCCTCTTGGCCGTGCGTCA
6.	Osterix	Forward -ACTGGCTAGGTGGTGGTCAG Reverse -GGTAGGGAGCTGGGTAAAGG

Table 2 Primers used for detection of precursor miRNAs in real time PCR in this study

S.No	miRNA	Sequence (5'-3')
1	mir-30-c-1	Forward-TGTGTAAACATCCTACAGTCTCAG Reverse-GAGTAAACAACCTCTCCCA
2	mir-222	Forward -GCTCAGTAGCCAGTGTAGA Reverse -AGACCCAGTACGCAGATGTA
3	mir-21	Forward -GCTTATCAGACTGATGTTGACTG Reverse -CAGCCCATCGACTGGTG

Western blot analysis

Whole cell lysates were prepared using RIPA lysis buffer (Bio Basic, Markham, Ontario, Canada) and the protein concentrations were determined by the Bradford method. The protein samples were loaded onto 12% polyacrylamide-SDS gels. After size resolution, the proteins were transferred electrophoretically to polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, California, USA). After blocking in Tris-buffered saline containing 5% (w/v) non-fat dry milk, the membrane was exposed to primary antibody overnight at 4 °C. The membrane was then washed and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive signals were visualized using an enhanced chemiluminescence detection kit (WESTAR Supernova; Cyanagen, Bologna, Italia) with Chemidoc instrument (Biorad, Hercules, California, USA). The blot was stripped and probed again with α -tubulin antibody for normalization.

Luciferase reporter assay

The wild-type 3' -UTR of Smad7 (sense: 5'-AACTAGCGCCGCTGTTTAGACTTTAA CATAAGCTAT-3', antisense: 3'-TTTGATCGCCGGCGACAAATCTGAAA TTGTATTC GATAGATC-5'), and mutant 3'-UTR of Smad7 (sense: 5' -AACTAGCGCCCGCTGTTT AGACTTTA ACATAATTTAT-3', antisense: 3'-TTTGATCGCCGGCG ACAAATCTGAA ATTGTATAAGATAGATC-5') were synthesized chemically. The nucleotides which are mutated are given in bold. Primers of sense and antisense containing an internal NotI site were annealed and cloned using PmeI and XbaI restriction sites which present in the dual-luciferase miRNA expression vector pmirGLO (Promega, Madison, WI). Cloned 3'-UTRs were present in downstream of the *Firefly* luciferase gene. *Renilla* luciferase was a constitutively expressed gene which present in the pmirGLO vector system. NotI digestion was performed to identify the clones. Cells were transiently transfected with 200 ng of the wild-type or mutant Smad7 3' -UTR constructs along with miR-21-5p mimic at a concentration of 50 nM(HMI0371, Sigma-Aldrich, St. Louis, Missouri, USA) or control miRNA by using Lipofectamine

2000 transfection reagent (Invitrogen, Grand Island, NY). After 24 h of transfection, whole cell lysates were collected in 1× passive lysis buffer, and luciferase activity was analyzed in a SYNERGY/HTX multi-mode reader (BioTek, Winooski, VT) using a dual luciferase reporter assay kit (Promega, Madison, WI). *Renilla* luciferase activity was used for normalization.

Statistical analysis

Statistical analysis was carried out using Student's *t*-test and one-way ANOVA. The *p* value of ≤ 0.05 was considered as significant comparing each of the group.

Results

SA at three different concentrations (100, 150, and 300 μ M) was used to check the cytotoxicity of SA to mMSCs. The results showed that cells treated with SA at the above concentrations had no change in OD value compared to control or untreated cells, suggesting the nontoxic nature of SA to mMSCs (Fig. 1).

Even though the anti-diabetic properties of SA have been reported (Muthukumaran et al. 2013), its effects on osteogenesis have not been determined. To discern the effects of SA on osteoblast differentiation at the cellular level, mMSCs were treated with SA at nontoxic concentrations (100, 150, and 300 μ M) for 7 d. ALP activity was determined using the BCIP-NBT assay. It was found that SA treatment at each of these concentrations increased ALP activity in mMSCs (Fig. 2A). The blue-violet staining of cells was quantified and the results showed a significant increase in ALP activity following SA treatment in these cells (Fig. 2B).

To further explore the potential nature of SA on osteoblast differentiation, Alizarin red and von Kossa staining were performed. Alizarin red staining results showed more calcium deposition when mMSCs were treated with SA for 14 d (Fig. 3A). The quantitative analysis of calcium deposits at 405 nm showed a significant increase in mineralization

(Fig. 3B). von Kossa staining also revealed more accumulation of calcium after SA treatment of mMSCs (Fig. 3C). A significant increase in calcium deposits was found at concentrations of 100 and 150 μ M SA in these cells (Fig. 3D). It appears that the effect of SA treatment at 150 μ M was found to be maximal during osteoblast differentiation of mMSCs (Figs. 2 and 3).

Since SA treatment of mMSCs promoted osteoblast differentiation at a cellular level (Figs. 2 and 3), we next investigated this effect at the molecular level. Osteoblast differentiation from MSCs is regulated by a complexity of transcription factors and signaling proteins, including Runx2, Osterix (Zhang 2010). Runx2 is a bone transcription factor and is essential for expression of osteoblast differentiation marker genes (Zhao et al. 2005; Javed et al. 2009; Karsenty et al. 2009; Muthusami et al. 2011; Vimalraj et al. 2014, 2015; Feng et al. 2016; Wu et al. 2017). To determine the effect of SA on Runx2 expression, mMSCs were treated with SA 150 μ M for 24 h. Total RNA and protein were isolated and subjected to reverse transcriptase qPCR (RT-qPCR) and western blot analyses, respectively. The results showed that SA treatment stimulated the expression of *Runx2* at the mRNA and protein levels in mMSCs (Fig. 4A and B). We noted that SA stimulated the expression of both *Runx2* mRNA and protein only at the 150 μ M concentration (Fig. 4).

Since Runx2 contributes a role in increasing expression of osteoblast differentiation marker genes (*ALP*, *Col-I*, *OC*) and SA treatment stimulated the expression of Runx2 maximally at 150 μ M concentration in mMSCs (Fig. 4), we next determined the effects of SA on the expression of osteoblast marker genes. mMSCs were treated with SA at 150 μ M for 7 and 14 d. Total RNA was isolated and subjected to RT-qPCR analysis. mRNA expression of *ALP*, *Col-I* and *OC* was significantly increased when mMSCs were treated with SA for 7 d (Fig. 5A, B and C). After 14 d of SA treatment, mRNA expression of *Col-I* and *OC* in mMSCs was significantly increased (Fig. 6A, B and C). Since Osterix is a downstream gene of Runx2 and is also involved in osteoblast differentiation (Zhang 2010), we determined its expression. The result showed that SA treatment for 7 and 14 d stimulated the expression of *Osterix* at the mRNA level in mMSCs (Figs. 5D and 6D).

It was evident from our results that SA stimulated expression of *Runx2* (Fig. 4) and this might be responsible for the promotion of osteoblast differentiation (Figs. 4, 5, and 6). There are reports indicating that Smad7, a co-repressor for Runx2, inhibits osteoblast differentiation (Valcourt et al. 2002; Eliseev et al. 2006; Iwai et al. 2008; Yano et al. 2012; Jia et al. 2014; Selvamurugan et al. 2017; Vishal et al. 2017b). Inhibition of Smad7 during osteoblast differentiation promoted Runx2 expression (Li et al. 2014; Vishal et al. 2017b; Wei et al. 2017; Selvamurugan et al. 2017). HDAC4 is another co-repressor that has been shown to negatively regulate Runx2

Fig. 1 Cytotoxicity assessment of SA. mMSCs were treated with SA at different concentrations (100, 150, and 300 μ M) for 48 h. The MTT assay was then performed as described in the methods section

Fig. 2 Effect of SA on ALP activity. **(A)** mMSCs were treated with SA at different concentrations (100, 150, and 300 μ M) in DMEM media for 7 d. The cells were then stained with BCIP-NBT reagent. Images were obtained using an inverted microscope. **(B)** Quantification of ALP activity done by the calorimetric method at 620 nm. *indicates a significant increase compared to control ($p < 0.05$)

expression/activity (Vega et al. 2004; Westendorf 2006; Jeon et al. 2006; Karsenty et al. 2009; Shimizu et al. 2010; Shimizu et al. 2014; Wang et al. 2014a, b; Bradley et al. 2015; Carpio and Westendorf 2016; Vishal et al. 2017a, b; Carpio et al. 2017; Xu et al. 2017). Hence, we next determined whether

SA treatment involves any changes associated with expression of Smad7 and HDAC4 during osteoblast differentiation (Fig. 7). mMSCs were treated with SA at different concentrations for 7 d and 14 d. Whole cell lysates were prepared and subjected to western blot analysis. We identified up- and down

Fig. 3 Effect of SA on Alizarin red and von Kossa staining of cells. **(A)** mMSCs were treated with SA at different concentrations (100, 150, and 300 μ M) in DMEM media for 14 d. The cells were then subjected to Alizarin red staining. **(B)** Quantification analysis of cells stained using Alizarin red. **(C)** mMSCs were treated with SA

at different concentrations (100, 150, and 300 μ M) in DMEM media containing osteogenic supplements for 14 d. The cells were then subjected to von Kossa staining. **(D)** Calorimetric estimation of von Kossa stained cells. *indicates a significant increase compared to control ($p < 0.05$)

Fig. 4 Effect of SA on the expression of *Runx2*. mMSCs were treated with SA at different concentrations (100, 150 and 300 μ M) in DMEM media for 24 h. **(A)** Total RNA was isolated, and cDNA was synthesized and subjected to RT-qPCR analysis using *Runx2* gene primers. *RPL13A* was used as an internal control for normalization. *indicates a significant

increase compared to control ($p < 0.05$). **(B)** Whole cell lysates were prepared and subjected to western blot analysis using Runx2 antibody. α -tubulin was used as an internal control for normalization. **(C)** Relative quantification analysis of biological triplicate samples. *indicates a significant increase compared to control ($p < 0.05$)

regulation of Runx2 and Smad7 proteins, respectively, by SA treatment after 7 d (Fig. 7A) and 14 d (Fig. 7B) treatments of mMSCs. The effect of SA treatment at 150 μ M was found to be maximal during osteoblast differentiation of mMSCs (Figs. 2 and 3) and at this concentration, there was no change in

HDAC4 protein level when cells were treated with SA for 7 (Fig. 7A) and 14 d (Fig. 7C).

To identify the molecular mechanisms responsible for down regulation of Smad7 by SA-treatment in mMSCs, we determined the expression of miRNAs. Three miRNAs

Fig. 5 Effect of SA on the expression of osteoblast differentiation marker genes and Osterix. mMSCs were treated with SA at 150 μ M in DMEM media for 7 d. Total RNA was isolated and subjected to RT-qPCR analysis using primers for **(A)** *ALP*, **(B)** *Col-I*, **(C)** *OC* and **(D)** *Osterix* genes. *RPL13A* was used as an internal control for normalization. *indicates a significant increase compared to control ($p < 0.05$)

Fig. 6 Effect of SA on the expression of osteoblast differentiation marker genes and Osterix. mMSCs were treated with SA at 150 μ M in DMEM media for 14 d. Total RNA was isolated and subjected to RT-qPCR analysis using the primers for (A) *ALP*, (B) *Col-I*, (C) *OC* and (D) *Osterix* genes. *RPL13A* was used as an internal control for normalization. *indicates a significant increase compared to control ($p < 0.05$)

Fig. 7 Effect of SA on the expression of Smad7 protein. mMSCs were treated with SA at different concentrations (100, 150, and 300 μ M) in DMEM media for (A) 7 d and (B) and (C) 14 d. Whole cell lysates were prepared and subjected to western blot analysis using antibodies against Smad7, HDAC4, Runx2 and α -tubulin. α -tubulin was used as an internal control for normalization

namely miR-222, miR-21, and miR-30c (Moorthi et al. 2013; Li et al. 2013; Lin et al. 2014; Yan et al. 2016a; Yoshizuka et al. 2016; Selvamurugan et al. 2017), which are known to target Runx2 corepressors, were selected for determining their precursor expression. Cells were treated with SA at 150 μ M for 14 d. Total RNA was isolated and subjected to RT-qPCR analysis for determination of expression of mir-222, mir-21 and mir-30c-1 in these cells. The result showed that SA treatment stimulated the expression of mir-21 and mir-30c-1 in mMSCs (Fig. 8A). There was greater stimulation of mir-21 by SA treatment for 14 d in these cells. We also found a significant increase in the expression of mir-21 by SA treatment for 24 h in mMSCs using RT-qPCR analysis (Fig. 8B) and this correlated with down regulation of Smad7 protein in these cells (Fig. 8C).

To identify that Smad7 is a direct target of miR-21-5p, a dual luciferase reporter assay system as reported earlier was used in the study (Vimalraj et al. 2014, 2017b). A region in the Smad7 3'UTR (1108–1129) was predicted to be the target of miR-21-5p using bioinformatics tool (Fig. 9A). The pmirGLO constructs containing the wild or mutant Smad7 3'-UTR downstream to the firefly luciferase reporter gene were transiently co-transfected along with control miRNA or miR-21-5p mimic in mMSCs. After 24 h, the luciferase activities of firefly and *Renilla* were measured. There was a significant decrease in luciferase activity when mMSCs were transfected with the pmirGLO containing Smad7 wild-type 3'-UTR along with the miR-21-5p mimic, whereas control miRNA had no

Fig. 8 Effect of SA on the expression of miRNAs. **(A)** mMSCs were treated with SA at 150 μM in DMEM media for 14 d. Total RNA was isolated, and cDNA was synthesized and subjected to qPCR analysis using the primers for mir-222, mir-21 and mir-30c-1. **(B)** mMSCs were treated with SA at different concentrations (100, 150 and 300 μM) in DMEM media for 24 h. Total RNA was isolated, and cDNA was synthesized and subjected to qPCR analysis using

the primers for mir-21. *RPL13A* was as used as an internal control for normalization. *indicates a significant increase compared to control ($p < 0.05$). **(C)** mMSCs were treated with SA at different concentrations (100, 150, and 300 μM) in DMEM media for 24 h. Whole cell lysates were prepared and subjected to western blot analysis using Smad7 and α-tubulin antibodies. α-tubulin was used as an internal control for normalization

significant effect on luciferase activity (Figs. 9 and 10). The mutant 3'-UTR of Smad7 was not significantly affected after transfection with the control miRNA or miR-21-5p, indicating the specificity of the 3'-UTR of Smad7 to miR-21-5p in these cells. Thus, this experiment provided conclusive evidence that miR-21-5p directly targets the 3'-UTR of the Smad7 gene.

Discussion

Osteoblast activity can be promoted by several drugs to treat skeletal disorders such as osteoporosis, but these drugs have potential harmful effects on human health due to their toxicity (Wissing 2015). Plant-derived polyphenols help in minimizing the toxicity to bone health (Pandey and Rizvi 2009; Tou 2015).

Fig. 9 **(A)** A region of wild or mutant Smad7 3' UTR binding site (1108–1129) with the seed sequence of miR-21-5p. **(B)** miR-21 directly targets Smad7. mMSCs were transiently transfected with the pmirGLO construct containing the wild-type or mutant Smad7 3'-UTR along with control miRNA or miR-21-5p. After 24 h, cell lysates were prepared and relative luciferase activity was calculated after normalization of firefly luciferase activity with *Renilla* luciferase activity. # indicates significant decrease compared to control miRNA ($p < 0.05$)

Fig. 10 Schematic diagram of SA action on mMSCs in osteoblast differentiation. SA treatment upregulated the expression of miR-21, and the increased miR-21 targeted *Smad7* expression, resulting in increased *Runx2* expression, thus leading to expression of osteoblast differentiation marker genes such as *ALP*, *Col-I*, and *OC* in mMSCs

It has been reported that SA exhibits anti-diabetic activity and has been used to treat diabetes in Chinese and Indian medicine (Wei et al. 2012a, b; Muthukumaran et al. 2013). Nontoxic behavior of SA is necessary for any type of cellular and molecular studies in mammalian cell lines. Hence, the toxicity of SA has been studied based on detection of blue-colored formazan by calorimetric methods using MTT reagent: the results demonstrated its nontoxic effects on mMSCs (Fig. 1). Even though a number of polyphenols have been reported for anti-diabetic, anti-oxidant and anti-cancerous activities, few have been tested for their activity on osteogenicity (Tseng et al. 2011; Gu et al. 2012; Al-Obaidi et al. 2014; Xiao et al. 2014; Fan et al. 2015; Du et al. 2017). Flavonoids such as Fisetin showed increased expression of *Runx2* and inhibited osteoclast differentiation (Leotoing et al. 2014). Flavonoids from *Tridax procumbens* directly promoted osteoblast differentiation by suppressing RANKL-induced differentiation of osteoclasts (Al Mamun et al. 2015). In our study, we reported that SA treatment promoted osteoblast differentiation at the cellular level, as evidenced by elevated ALP activity and increased accumulation of calcium deposits in mMSCs (Figs. 2 and 3).

Runx2 is a bone transcription factor which is essential for the promotion of expression of osteoblast differentiation marker genes such as *ALP*, *Col-I* and *OC* (Zhao et al. 2005; Javed et al. 2009; Karsenty et al. 2009; Muthusami et al. 2011; Vimalraj et al. 2014, 2015; Feng et al. 2016; Wu et al. 2017). A defect in the expression of *Runx2* leads to skeletal abnormalities associated with bone diseases (Lou et al. 2008; Cardoso et al. 2010; Shibata et al. 2015; Sun et al. 2016; Zhang et al. 2017; Bir et al. 2017; Chen et al. 2017a, b; Zeng et al. 2017; Jung et al. 2017). The analysis of osteoblast differentiation at the molecular level by the SA treatment in mMSCs showed the stimulation of *Runx2* expression in these cells (Fig. 4). Others, such as resveratrol, hyperoside, baicalin, kaempferol, linarin, and formononetin, also promoted high levels of *Runx2* mRNA expression in osteoblastic cells (Tseng et al. 2011; Guo et al. 2011; Guo et al. 2012; Zhang et al. 2014; Li et al. 2016;

Singh et al. 2017; Gaoli et al. 2017; Wu et al. 2017). Upon treatment with flavonoids, *Runx2* levels increased, which promoted the expression of osteogenic genes in cells. Quercetin, a flavonoid, stimulated ALP, OC, OPN, BMP-2, BSP expression in bone marrow-derived mesenchymal stem cells (Kim et al. 2006; La et al. 2013; Zhou and Lin 2014). In our study, the stimulation of *Runx2* expression by SA treatment in mMSCs correlated with the expression of ALP, Col-1, and OC at 7 d (Fig. 5) and at 14 d (Fig. 6).

We further dissected the molecular mechanisms responsible for SA-stimulated osteoblast differentiation in mMSCs. Flavonoids are known to stimulate osteoblast genes via various signaling cascades. Isoflavones (Syringetin, Genistein, Linarin, hesperetin and sulfuretin) have been shown to promote osteoblast differentiation via upregulation/activation of BMP2/SMAD5/Akt/*Runx2* pathways (Hsu et al. 2009; Trzeciakiewicz et al. 2010; Dai et al. 2013; Li et al. 2016; Auh et al. 2016). The TGF- β /BMP signaling pathway involves the activation of type I and type II serine threonine kinase receptors, which phosphorylate intracellular Smad proteins. Receptor or regulatory Smads, such as Smad-1, -2, -3, -5, and -8, bind to Smad-4 (common or co-Smad). They undergo nuclear translocation and initiate the transcription of target genes (Selvamurugan et al. 2007; Horbelt et al. 2012; Chen et al. 2012; Crane and Cao 2014; Arumugam et al. 2017; Selvamurugan et al. 2017). This pathway in turn can be affected by a negative feedback loop controlled by inhibitory Smads such as Smad-6 and -7 (Ishida et al. 2000; Yan et al. 2009; Kamiya et al. 2010; Yano et al. 2012; Yan et al. 2016b). *Runx2* corepressors such as HDAC4, Smurf1, and Smad7 that negatively regulate osteoblast differentiation (Zhao et al. 2004; Hug 2004; Shimizu et al. 2010; Obri et al. 2014; Li et al. 2014; Shimazu et al. 2016; Vishal et al. 2016; Vishal et al., 2017a, b; Wei et al. 2017) were identified. The expression of Smad7 as one of the *Runx2* corepressors was found to be decreased by the SA treatment in mMSCs (Fig. 7), which suggested the involvement of the TGF- β /BMP signaling pathway for SA-stimulated osteoblast differentiation in these cells.

Several miRNAs have been identified as positive (miR-20a, 27, 29c, 196a, 210, 29-b, 15b, 433-3p, 215, 335-5p, 378, 2861) and negative (miR-23a, 26a, 100, 103a, 133, 138, 132, 135, 182, 204, 211, 335, 637) regulators (Lian et al. 2012; Wei et al. 2012a, b; Zheng et al. 2012; Kim et al. 2012; Vimalraj and Selvamurugan 2012; Vimalraj and Selvamurugan 2015; Moorthi et al. 2013; Hu et al. 2015; Zuo et al. 2015; Tang et al. 2017) during skeletal development, indicating that miRNAs act as regulators of osteogenesis. The reasons for selecting three miRNAs in this study were as follows: expression of osteoblast marker genes can be up-regulated by down regulation of miR-222 (Yoshizuka et al. 2016), and miR-21 and miR-30c-1 have been reported to target Smad7 and HDAC4, respectively. The expression levels of both miR-21 and miR-30c-1 were stimulated by the SA

treatment in mMSCs (Fig. 8A) but the fold change was higher with the expression of miR-21 (Fig. 8B) with down regulation of Smad7 expression (Fig. 8C). Smad7 was also identified as a direct target of miR-21 using the luciferase reporter system in mMSCs (Fig. 9). There are evidences indicating that miR-21 targets Smad7 in several types of cells (Liu et al. 2010; Lin et al. 2014; Wang et al. 2014a, b; McClelland et al. 2015; Yang et al. 2016; Selvamurugan et al. 2017). We recently reported that the targeting of Smad7 by miR-590-5p resulted in stabilization of *Runx2* during osteoblast differentiation (Vishal et al. 2017b). Thus, the decreased expression levels of Smad7 by miR-21 could be correlated with increased expression/stabilization of *Runx2* in mMSCs by SA treatment. Therefore, it is more likely that, as a result of SA treatment, there was increased expression of miR-21 which may inhibit Smad7 expression so that expression of *Runx2* increased, which led to increased osteoblast differentiation (Fig. 10).

Overall, based on our results, we suggest that SA is potentially involved in the promotion of mMSCs into osteoblasts, and hence it may be considered as an osteogenic compound to treat various bone and bone-related diseases in the future.

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