

MODULATION OF POLYAMINES TO ELUCIDATE FUNCTION WITHIN
OSTEOGENESIS OF MESENCHYMAL STROMAL CELLS

By

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ABSTRACT

MODULATION OF POLYAMINES TO ELUCIDATE FUNCTION WITHIN OSTEOGENESIS OF MESENCHYMAL STROMAL CELLS

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Snyder-Robinson Syndrome (SRS), an X-linked intellectual disability that arises in children, exhibits debilitating phenotypes like severe osteoporosis. These patients demonstrate an inability to produce mineralized new bone in comparison to the rate at which bone is resorbed, thus leading to weaker skeletal structure and atraumatic fractures. The known cause of SRS is due to loss-of-function mutations within the gene that encodes Spermine Synthase (SMS). Loss of SMS enzymatic activity, which catalyzes the conversion of polyamines spermidine into spermine, demonstrates an increase in the spermidine/spermine ratio in all documented cases of this disorder. The involvement of polyamines in osteogenesis is still not well understood, but it is apparent that maintaining strict regulation of these organic compounds is necessary for bone mineralization. Due to deficient therapeutic intervention, it is imperative to continue researching the molecular mechanisms by which the polyamine pathway regulates bone mineralization. Here we investigated the effects of exogenous supplementation of either putrescine, spermidine, or spermine throughout osteogenic differentiation within healthy human bone-marrow derived mesenchymal stromal cells (MSCs). Through this, we confirmed the ability to transport polyamines within MSCs, as well as observed inhibitory effects throughout osteogenesis. We found that excess spermidine inhibits alkaline phosphatase activity, an important osteogenic marker, while excess spermine decreases hydroxyapatite synthesis

as confirmed through Alizarin red S staining. Furthermore, we observed the gene that encodes spermidine/spermine N1-acetyltransferase 1 (SAT1) depicts a powerful upregulation in expression at the mRNA level during osteogenic differentiation in healthy MSCs. To further explore this, we silenced SAT1 expression using lentiviral vectors expressing a SAT1-specific shRNA. Our preliminary studies suggest that suppressing SAT1 expression has minimal effect on osteogenesis. These data suggest that the conversion of higher polyamines into more easily excretable lower polyamines is necessary in bone development, and altogether, our studies propose that SRS is due to the accumulating spermidine interactions within cells throughout osteogenesis.

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INTRODUCTION

Snyder-Robinson Syndrome is a rare X-linked intellectual disorder that phenotypically arises as kyphoscoliosis, muscle mass deficiency, facial dysmorphism, seizures, and osteoporosis (OMIM #390583). First described in 1969 (Snyder, 1969), this syndrome presents itself with developmental delay and motor disability, leading to failure of meeting developmental milestones (Schwartz, 2013). Patients with SRS suffer from atraumatic fractures frequently due to reduced cancellous and cortical bone (Schwartz, 2013). Current treatments for SRS follow regimens similar to most osteoporosis therapeutic interventions, however therapies such as bisphosphonates and calcium supplementation show only moderate improvement. Therefore, it is important to elucidate SRS disease-specific osteoporotic pathology to improve upon current therapies.

The debilitating effects observed within SRS are due to loss of function mutations within the gene encoding spermine synthase (*SMS*) on human chromosome Xp22 (Pegg, 2014). The *SMS* enzyme catalyzes the conversion of spermidine (SPD) into spermine (SPM) through the transfer of an aminopropyl group (Figure 1; Proietti, 2020; Murray-Stewart, 2020). Deficient *SMS* activity leads to a lack of spermine while exhibiting an aggregation of spermidine. Previous studies have attempted to ameliorate the lack of spermine in patients with SRS through supplementation and injection, however poor uptake of spermine lead to unsuccessful restoration (Wang, 2009).

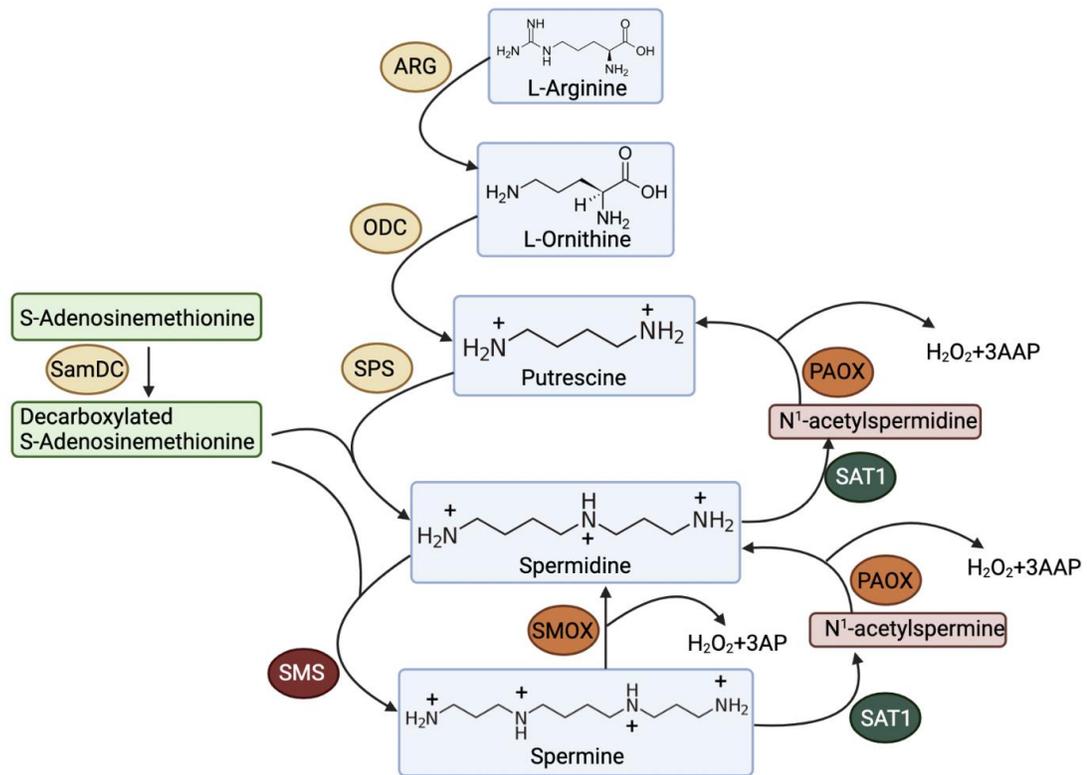


Figure 1: Biosynthesis of polyamines within mammals.

Polyamines, consisting of ornithine, putrescine (PUT), spermidine, and spermine, are ubiquitously found in almost every cell type of eukaryotic organisms, though only the latter three are synthesized in mammals (Pegg, 2009). These polycations play indispensable roles within cellular function and interact with negatively charged molecules like chromatin, phospholipids, nucleic acids, ion channels and specific proteins (Murray-Stewart, 2018). Polyamines are generated within mammals through biosynthesis, gut microbiota, and ingestion. Biosynthesis of the triamine spermidine and the tetraamine spermine is carried out by aminopropyltransferases designated as spermidine synthase (SRM) and spermine synthase (SMS) respectively (Pegg, 2009). Polyamine regulation is a complex and highly regulated process in which oxidases and

acetyltransferases work in reverse of aminopropyltransferase reactions to convert higher polyamines into the more readily excreted putrescine (Figure 1; Pegg, 2009). The enzyme spermidine/spermine-N¹-acetyltransferase 1 (SAT1), acetylates both spermidine and spermine, synthesizing N¹-acetylspermidine or N¹-acetylspermine, respectively (Figure 1). These molecules are then either exported out of the cell directly or oxidized by polyamine oxidase (PAOX) into lower polyamines.

Though polyamine imbalances are the cause of osteoporosis within SRS patients, it is still unclear whether this phenotype is due to the dysregulation of one or both polyamines (Albert, 2015). Studies have shown that SAT1 expression increases with supplementation of polyamines as well as the induction of adipogenesis and osteogenesis, which in turn would convert spermidine and spermine into the more excretable acetylated versions (Tsai, 2015). This would suggest that the excretion of spermine and spermidine allows for osteogenic differentiation to occur. In contrast, there have been reports stating supplementation of spermine or spermidine have increased osteogenesis (Facchini, 2012; Guidotti, 2013; Tjabringa, 2008). With discordant conclusions regarding the role of polyamines in osteogenesis, it is critical to shed further light onto this molecular mechanism.

For the past decade, the efficacy of bone marrow derived MSCs in skeletal tissue regeneration has been examined due to their multipotent differentiation capabilities, immune-modulatory, and anti-inflammatory properties (Arthur, 2020). In order to develop bone *in vivo*, bone minerals composed of hydroxyapatite crystals (inorganic component) and collagen type I dependent extracellular matrix (organic component), are

produced by bone forming osteoblasts (Arthur, 2020; Hana, 2018). MSC differentiation into osteoblasts has been performed since the late 1960s-early 1970s (Friedenstein, 1968; Friedenstein, 1970). The ability of MSCs to recapitulate bone mineralization *in vitro* allows for the exploration of skeletal developmental defects. Previous reports show SRS patient-derived human bone marrow mesenchymal stromal cells (MSCs) exhibit a severe deficiency in osteogenic differentiation capability as well as calcium phosphate mineralization (Albert, 2015). This lack of proliferative and osteogenic potential has also been modeled within healthy MSCs utilizing an shRNA lentiviral vector knocking down *SMS* expression (Ramsay, 2019), suggesting basal *SMS* activity is crucial for proper differentiation and validating the use of MSCs in recapitulating SRS phenotype *in vitro*.

The goal of this study was to uncover the role of polyamine homeostasis in osteogenesis. Due to inconsistent results regarding the response to polyamines in the context of osteogenesis, it is unclear whether the excess or dearth of polyamines promotes bone formation. By manipulating polyamine homeostasis during MSC osteogenic differentiation, we were able to observe the role of polyamines within that process. MSCs derived from multiple donors were cultured in osteogenic differentiation media supplemented with polyamines (putrescine, spermidine, and spermine). To better understand the role of SAT1 in this process, we silenced the gene using an shRNA lentiviral vector. All of this together gave us insight into the physiological increase of spermidine observed within patients with SRS, as well as the response to excess putrescine and spermine. The work presented here provides clearer data with higher rigor of the response to polyamines during osteogenesis *in vitro*. Here, we gained further

knowledge of the role of SAT1 in bone forming, as well as the response to excess exogenous polyamines within MSCs during differentiation.

METHODS

MSC Isolation and Expansion

MSCs were isolated from fresh bone marrow aspirates collected from the posterior iliac crest, which are commercially available (StemExpress, Sacramento, CA, USA). Bone marrow was derived from healthy male and female donors, where the age ranged from 20-45 years old. Bone spicules were isolated by passing bone marrow through a 90 μ M pore strainer, aspirates were diluted in equal volume of phosphate-buffered saline (PBS), and centrifuged at 700 x g for 30 minutes over Ficoll. Bone spicules and mononuclear cells collected from the buffy coat were plated using Minimum Essential Medium α (MEM α -HyClone, Logan, UT, USA) containing 10% Fetal Bovine Serum (FBS-Pheonix Scientific, San Marcos, CA, USA) within plastic flasks. Non-adherent cells were removed by washing three times with PBS after two days in culture. For each experiment, MSCs between passage 3 and 6 were used.

Generation of Lentiviral Vectors and Transduction

Third generation lentiviral constructs were used with the form: pCCLc-U6-shRNA-PGK-tdTomato-WPRE, in which the shRNA is either the sequence to silence SAT1 (shSAT1), or an shRNA sequence that does not target any human gene (shControl). The sequence of the mRNA-targeting portion (RNAi) of shSAT1 is 5'-CAGAAATTCTGAAGAATCTAAGCCA-3'. For two flasks, the packaging plasmids VSVG (10 μ g) and Δ 8.91(50 μ g), along with the plasmids of interest (50 μ g), were used

to transfect Lenti-X 293 T cells using TransIT-293 Transfection reagent (Mirus Bio, cat# MIR 2705). An epi-fluorescence microscope measured tdTomato positive cells on the third day following transduction with protamine sulfate (20 µg/ml). Lentiviral volumes generating approximately 80% transduction efficiency were deemed sufficient for further experiments.

Gene Expression

Total RNA was extracted at specified time points using Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA), adhering to the manufacturer's protocol. Taqman reverse transcription reagents (Invitrogen, Irvine, CA, USA) were used to reverse transcribe extracted RNA, synthesizing cDNA for each sample. mRNA levels of alkaline phosphatase, spermine synthase, N1-acetyltransferase 1, N1-acetyltransferase 2, spermine oxidase, and integrin binding sialoprotein were measured using Taqman Universal Master Mix reagents (Invitrogen) and Taqman gene expression assays (Invitrogen). Probes can be found using the following Assay ID: GAPDH: Hs03929097_g; SAT1: Hs00971739_gl; SMS Hs01924834_ul; SMOX: Hs 00602494_ml; IBSP: Hs00173720_m1; ALPL: Hs01029144_m1. mRNA levels of osteocalcin and osteopontin were measured using SYBR Green expression methods (Invitrogen) and PowerUp SYBR Green Master Mix reagents (Applied Biosystems). Primers can be identified by the following ID: Osteopontin (OPN): Fwd: CTCAGTGTAGCCCAGGATGC Rev: ACCACCATGGAGAAGGCTGG; Osteocalcin (OCN): Fwd: GACTGTGACGAGTTGGCTGA Rwd: CTGGAGAGGAGCAGAACTGG.

Cell Proliferation Assay

MSCs were seeded at 10,000 cells per well in 12-well plates. After 24 h (Day 0) and at day 3 MEM α + 10% FBS containing 1mM aminoguanidine HCl (cat# 396494) was added with or without supplements. Cells were treated with 10 μ M spermine (cat# 55513), spermidine (cat# 5262), or putrescine (cat# 51799), with one medium change on day 3. On day 0, 2, 4 and 6 MSCs were lifted using trypsin and counted using Trypan blue exclusion dye and hemocytometer.

Osteogenic Differentiation Assays

Osteogenic differentiation was performed as previously described (Templeton, 2021). Briefly, MSCs were seeded at an initial density of 15,000 cells/cm² and received regular media changes every 3-4 days. Cells were cultured within two different experimental conditions. Cells were cultured with osteogenic differentiation medium containing 1mM aminoguanidine HCl. The cells were treated with or without 0.1 μ M, 1.0 μ M, or 10 μ M of either spermine, spermidine or putrescine. These cells were transduced with lentiviral vectors (see Generation of Lentiviral Vectors and Transduction within the Methods section) containing either shC or shSAT1 and received regular media changes with standard osteogenic medium. Standard osteogenic differentiation medium is composed of MEM α + 10%FBS supplemented with 0.2 mM ascorbic acid, 20 mM β -glycerophosphate, and 0.1 μ M dexamethasone.

Matrix mineralization was determined using Alizarin Red S indicator (ARS; Ricca Chemicals, Arlington, TX, USA) following 21 days of differentiation. Cells were

fixed for 15 minutes in 10% *v/v* formalin solution, washed with PBS, and stained with 1% *w/v* ARS for 20 minutes with gentle shaking. Samples were then washed twice with PBS and twice with molecular grade water and photographed for visual determination. Samples were then incubated with 10% acetic acid and gentle shaking for 30 minutes. The cell layer was scraped off and the solution was collected, vortexed for 30 seconds, centrifuged at 14,000 rpm for 10 minutes. The supernatants were collected, and the optical density was measured at 405 nm on a spectrophotometer.

Alkaline phosphatase activity was measured following 10 days within osteogenic differentiation media regardless of experimental conditions. Samples received 200 μ l alkaline phosphatase lysis buffer (Tris-HCl + 1% Triton X-100), and the cells layer was scraped off and collected, vortexed for 30 second, placed on a vigorous shaker in ice for 15 minutes, vortexed for 30 seconds, and centrifuged at 14,000 rpm 4°C for 5 minutes. The supernatant was plated in triplicate on a 96-well plate in which p-nitrophenyl phosphate (PNPP; Thermo Fisher) was added, incubated in the dark at 37°C for 10 minutes, and the optical density was measured at 405 nm. To normalize these experiments, protein content was measured by adding Coomassie blue (cat# B0770) and measuring optical density at 595 nm.

Western Blotting

Approximately 1 million MSCs were lifted using Trypsin treatment. Cells were then lysed in ice-cold RIPA buffer (Thermo Fisher, cat# 89900) containing a protease and

phosphatase inhibitor cocktail (Thermo Fisher, cat# 78440). Cell lysates were mixed with Laemmli buffer (Bio-Rad, cat# 1610747) containing β -mercaptoethanol, and boiled at 95 °C for 5 minutes, then subjected to SDS–PAGE. Proteins were transferred onto PVDF membranes and incubated overnight with primary antibodies against SAT1 (1:2,000, Cell Signaling, cat# 61586) and β -actin (1:2,000, Santa Cruz, cat# sc-47778). Secondary antibodies (1:5,000) conjugated to horseradish peroxidase (Santa Cruz, cat# sc-2005) were then added for 1 hour at room temperature, and proteins were detected and photographed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, cat# 34580) and Image Lab software (Bio-Rad). Semi-quantification of protein levels was calculated by dividing pixel intensity of SMS by β -actin, measured using Adobe Photoshop CS6 software.

Statistical Analysis

The presented results are represented as the average with error bars derived from the standard error of mean (SEM). MSCs were derived from different donors, where n represents biological replicates in respective figure legends. Each experiment was executed at least 4 times unless otherwise noted. Depending on the number of comparable conditions, statistical significance was determined using 1-way ANOVA, followed by post hoc Tukey test, or a paired Student's t test.

RESULTS

Polyamine supplementation inhibits osteogenesis without signs of toxicity

To elucidate the role of polyamines within osteogenesis, we began by supplementing different concentrations of putrescine, spermidine, and spermine into the media of healthy bone-marrow derived MSCs (Figure 2). Compared to controls, alkaline phosphatase activity, an early osteogenic marker, was significantly decreased ($p < 0.0005$) only with supplementation of 10 μM spermidine into the osteogenic media (Figure 2A). A strong decrease in mineralization occurred with addition of both spermidine and spermine following 21 days in osteogenic media (Figure 2C). However, when quantifying these results, significant inhibition ($p < 0.05$) was only detected within the high concentration of spermine treatment (Figure 2B). Following these experiments, we tested if addition of exogenous polyamines exhibits a toxic effect due to the reactive aldehydes, such as acrolein and hydrogen peroxide, produced in catabolic oxidase reactions (Pegg, 2013). When supplementing the high concentration of each polyamine (10 μM), we observed minimal change in proliferation over 6 days within any condition compared to control (Figure 2D). These data suggest that MSCs can take up polyamines when introduced through medium, as well as the effects on different osteogenic stages. The results suggest that contrary to other literature sources (Facchini, 2012; Guidotti, 2013; Lee, 2013), both spermidine and spermine, though not toxic, show an inhibitory effect on osteogenesis within MSCs when supplemented into media at a concentration of 10 μM .

To understand if there is a transcriptional reaction to polyamine supplementation within enzymes related to the polyamine pathway, we measured mRNA utilizing RT-PCR following two days in standard MSC media with 10 μ M supplementations (Supplementary 1). From this, we observed no change in expression of polyamine enzymes compared to control, suggesting the changes observed in osteogenesis is due to excess polyamines rather than increased enzymatic expression.

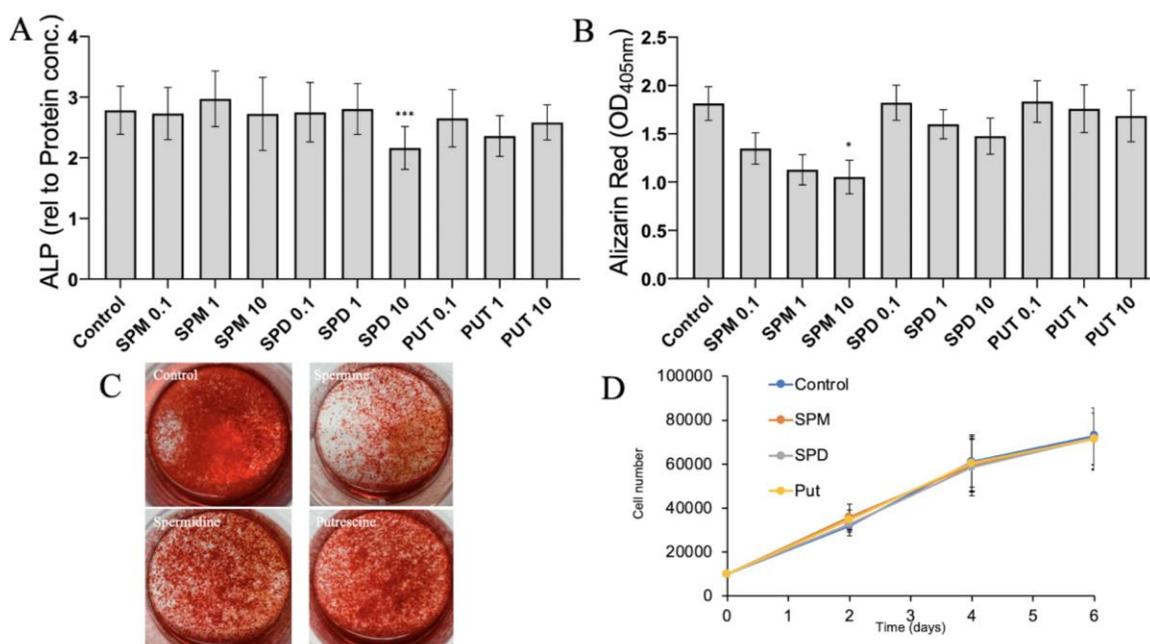


Figure 2: Supplementation of polyamines show inhibitory effect on osteogenesis without compromising proliferative potential. (A) Alkaline phosphatase activity relative to protein level following 10 days in osteogenic media with continuous supplementation of either SPM, SPD, or PUT at 0.1, 1.0, and 10 μ M (n=7) (B) Alizarin Red S staining measured after 21 days in osteogenic media (n=6). (C) Representative images of Alizarin Red S staining from MSCs after 21 days in osteogenic media, supplemented with either SPM, SPD, or PUT supplementation (10 μ M). (D) Proliferation curve with cells treated with 10 μ M SPM, SPD, or PUT (n=6). Error bars represent standard error of the mean. Statistical differences were calculated using 1-way ANOVA (followed by post hoc

Turkey test), where $*p < 0.05$ and $***p < 0.0005$. Data points not labeled are non-significant (n.s.).

SAT1 activity is a key regulatory enzyme throughout osteogenic differentiation

To further understand how polyamines are regulated throughout osteogenesis, expression of key enzymes and osteogenic markers throughout differentiation must be understood. Previous studies have shown that SAT1 expression is upregulated within osteogenic and adipogenic differentiation media (Tsai, 2015; Lee 2014), which we confirmed (Supplementary 2). To map out key polyamine enzymes (SMS, SAT1, and SMOX) and osteogenic markers (ALPL and IBSP), mRNA expression was measured at regular intervals throughout osteogenesis utilizing real-time PCR (Figure 3A). Though gene expression within most of these were quite varied amongst donors (Figure 3A; Supplementary S2), SAT1 consistently displayed significant increases in mRNA expression throughout the duration of osteogenesis ($*p < 0.05$; $**p < 0.005$). The upregulation of SAT1 protein during osteogenesis was confirmed by Western blot (Figure 3B). Expression of SMOX, which was one of our original enzymes of interest as the inverse reaction of SMS, was expressed at much lower levels throughout osteogenesis, shifting focus to the role of SAT1 in osteogenesis. Though more replicates

are required, these data suggest SAT1 plays a key role in bone mineralization.

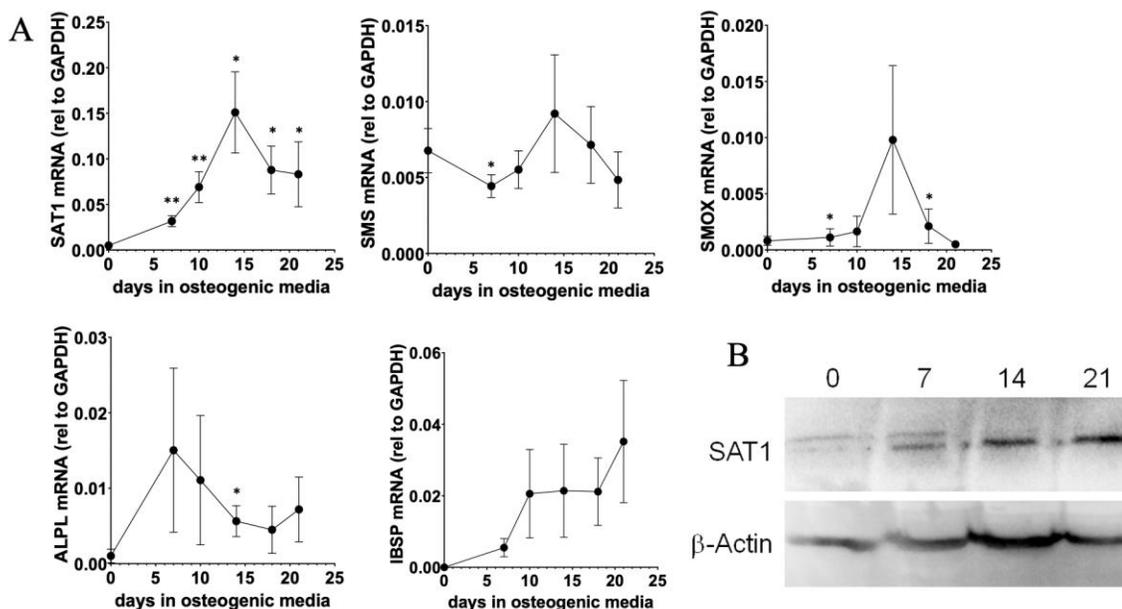


Figure 3: SAT1 expression increases throughout osteogenesis. (A) Real time PCR for different polyamine pathway enzymes and osteogenic genes (n=6). (B) Representative image of SAT1 protein levels throughout osteogenesis, where numbers indicate days in osteogenic media. Repetitions for **quantitative** analysis are pending. Error bars represent standard error of the mean. Statistical differences were calculated with a student's t-test, where * $p < 0.05$ and ** $p < 0.005$. Data points not labeled are non-significant (n.s.).

Preliminary data knocking down SAT1 suggests compensatory mechanism for polyamine regulation

To silence SAT1 expression, we transduced MSCs with lentiviral vectors designed to either express an shRNA that does not bind to any human transcript (shControl), or an shRNA that blocks the translation of *SATI* (shSAT1) (Figure 4). We verified the reduction of SAT1 at the mRNA level in comparison to shControl (Figure 4B). Alkaline phosphatase activity was used to monitor the early osteogenic developmental pathway and we observed that there is almost no change between shControl and shSAT1 (Figure 4C). Here, we expected that silencing SAT1 would lead to

an accumulation of both spermidine and spermine, mimicking the excess polyamines presented in the earlier experiments, thus leading to decreased osteogenic potential, however this is not what was observed. Further confirmation through shSAT1 mRNA expression throughout osteogenesis, and HPLC observing polyamine levels in response to shSAT1 are necessary to validate shSAT1 knockdown over time as well as the effects on polyamines. Consequently, as shown in Fig. 4D there was also minimal effect when measuring mineralization following transduction with either shControl or shSAT1. If the results of the alkaline phosphatase activity assay and alizarin red s staining are true, this suggests there may be a compensatory mechanism in which the polyamines are regulated to exhibit a typical osteogenic phenotype. Another explanation is that the knockdown of SAT1 is not maintained throughout osteogenesis. Regardless, these results are preliminary, and require further experimentation to arrive at any conclusions.

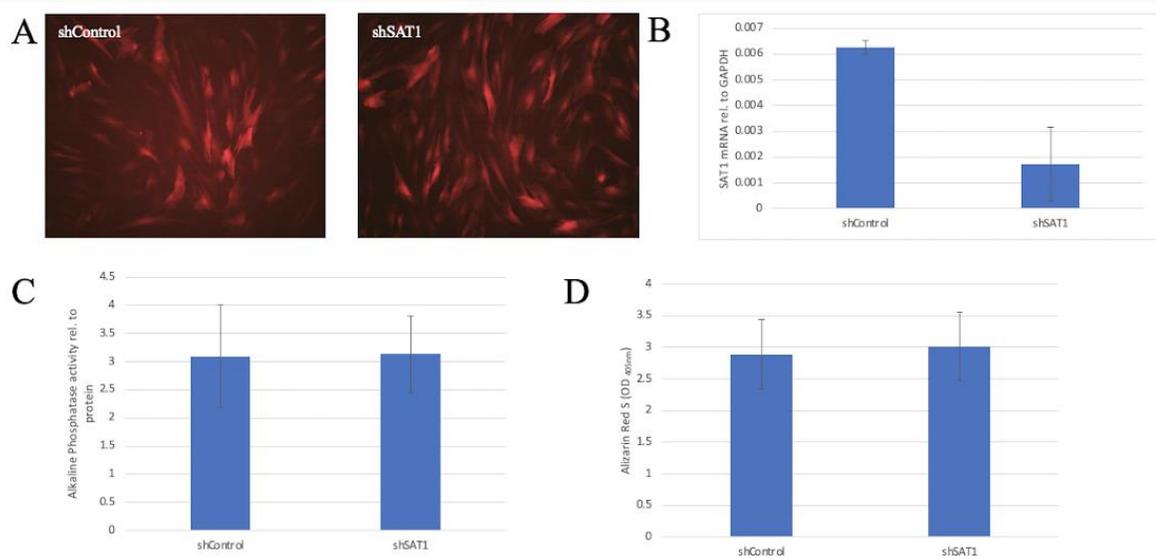


Figure 4: Deficiency in SAT1 depicts compensatory mechanism regulating polyamines. (A) Representative images of MSCs transduced with either shControl or shSAT1. (B) Real time PCR of MSCs transduced with either shControl or shSAT1 (n=2). (C) Alkaline phosphatase activity of MSCs transduced with either shControl or shSAT1 (n=3). (D) Quantification of Alizarin Red S stains of MSCs transduced with either shControl or shSAT1 (n=3). Error bars are represented as the standard error of the mean. Data points not labeled are non-significant (n.s.).

DISCUSSION

Amongst the multitude of debilitating phenotypes that arise within SRS patients, osteoporosis leads to a dramatic decrease in quality of life through common atraumatic fractures (Albert, 2015). This project sought to further clarify the role of polyamines within the context of bone formation. To explore this topic, we tested the effects of polyamine supplementation within MSCs isolated from multiple donors in an attempt to interpret a consistent pattern of polyamine expression required for differentiation. Polyamine supplementation had an inhibitory response on both an early stage of osteogenesis, alkaline phosphatase activity, and the late stage of hydroxyapatite synthesis. Spermidine supplementation showed a significant inhibition of alkaline phosphatase activity, and while not significant, an inhibitory trend of bone formation was also seen. Interestingly, and contrary to previous studies, we observed a significant inhibition of bone mineralization with the supplementation of spermine. We also examine the role of SAT1 throughout osteogenesis within MSCs, depicting a strong and consistent upregulation at both the mRNA and protein level when cultured in osteogenic media, suggesting its importance in bone development. Our initial attempt at knocking down SAT1 through shRNA targeting did not have a significant effect on either alkaline phosphatase activity or mineralization.

Supporting the notion that an increase in spermidine:spermine caused by SRS leading to an accumulation of spermidine, our work suggests that there is an inhibitory response to osteogenesis with an excess of polyamines that is not due to a decrease in

cellular proliferation. This may be due to a multitude of interactions polyamines are known to take part in. These polycations interact with many different cellular homeostatic functions, such as gene regulation, hypusination of eIF5A, regulation of ion channels, carbonic anhydrases, and glutamate receptors (Pegg, 2010). Though a significant decrease of osteogenesis is observed with the addition of exogenous polyamines, it is still unclear if this recapitulates physiological conditions and further experimentation is needed. To further support these data, measuring polyamine contents through high performance liquid chromatography would provide further insight into the uptake of polyamines. Measuring transcriptional changes of polyamine enzymes as well as osteogenic markers following supplementation of polyamines within osteogenic differentiation media would also give further clarity to the response of excess polyamines within MSCs. Though, *in vitro* data can tell us only so much. Furthermore, *in vivo* studies utilizing a conditional SRS knockout mouse through observation of polyamine levels in different tissues, restoring SMS activity through associated-adenovirus delivery, or overexpression of SAT1 could reveal physiological polyamine levels within SRS.

Future endeavors to further understand polyamine function within bone mineralization require observing the relationship between polyamines, ion channels, and carbonic anhydrases. Spermidine, though at lower efficiency than spermine, has been shown to block inward rectifying potassium channels (Kir) as well as inhibit carbonic anhydrases (Pegg, 2010; Carta, 2010; Scozzafava, 2016). Pini et al. have shown that chondrogenic and osteogenic genes are reliant on Kir 2.1 function, which is markedly inhibited by both spermidine and spermine (Baronas, 2014; Pini, 2018). This could be

investigated by observing ion channel expression throughout osteogenesis to determine key ion channels for bone formation. Following this, measuring ion channel currents in response to excess polyamines would determine potential inhibition. Unfortunately, current technology does not allow the visualization of specific polyamines, however polyamineRED (DioagnoCine) indiscriminately stains all polyamines. Co-immunostaining ion channels of interest with polyamineRED throughout osteogenesis and within SRS model MSCs could also give further clarification regarding the presence of polyamines in relation to ion channels. Carbonic anhydrases (CA), a group of isozymes that reversibly convert carbon dioxide into bicarbonate, plays a key role in hydroxyapatite synthesis (Adeva-Adany, 2015). Carbonic anhydrases have also been shown to be inhibited by the higher polycations spermidine and spermine (Scozzafava, 2016). CA transcriptional activity throughout osteogenesis is still not yet understood. Elucidating expression of CAs during osteogenesis, as well as inhibition of CAs could shed further light onto the interactions of polyamines and CAs in the context of bone formation. Similarly, staining polyamines as well as CAs of interest could reveal valuable information regarding spatial interactions. Either of these mechanisms presents a potential path as to how polyamine imbalance affects bone mineralization and would be fascinating to explore.

Through measuring mRNA of polyamine enzymes within MSCs throughout osteogenesis, we show a substantial increase in SAT1 mRNA and protein. This increase in SAT1 suggests that more easily excretable acetylated polyamines, or the conversion of higher polyamines into the also more easily excreted putrescine, is crucial for proper

osteogenesis. Supporting the concept that polyamine excretion is necessary for proper ion channel function, DeHart et al. showed that SAT1 enhances $\alpha 9\beta 1$ integrin mediated migration through catabolism of spermine and spermidine, decreasing Kir4.2 blockage (DeHart, 2008). The mechanism explored in this publication concluded that SAT1 regulated Kir4.3 function through the acetylation of polyamines. Acetylated spermine or spermidine are more easily excreted and removal of these molecules frees Kir4.2, allowing colocalization with $\alpha 9\beta 1$ in focal adhesions at the leading edge. This study further enhances the idea that polyamine mediated regulation of ion channels may be the cause of impaired osteogenesis. Specifically in SRS, spermidine accumulation may be causing a blocking of necessary ion channels. Though as mentioned previously, the relation of polyamines and ion channels in the context of bone formation is still yet to be explored.

Though the addition of exogenous polyamines may give insight into the response to excess polyamines within MSCs, there may be dissimilarities in reaction when compared to intrinsic polyamines derived through the biosynthetic pathway. To explore this, we also observed osteogenesis following the knockdown of an essential polyamine catabolic enzyme, SAT1. Preliminary data regarding SAT1 deficiency appears to have minimal effect on osteogenesis *in vitro* (Figure 4C and Figure 4D). This does not support our initial hypotheses, which predicts the knockdown of SAT1 would result in a decrease in osteogenic capability. This would render the MSCs incapable to acetylate and excrete higher polyamines efficiently, thus mimicking exogenous supplementation of excess

polyamines. These data suggest that a compensatory mechanism in place of SAT1 function, potentially spermine oxidase (SMOX), is upregulated in response, though preliminary transcriptional expression of SMOX appeared to be low in comparison to SAT1 in the context of osteogenesis. Conversely, SMOX function is only capable of converting spermine into spermidine, but there is not an enzyme that catalyzes the deamination of spermidine into putrescine directly. Another potential compensatory mechanism is the downregulation of ornithine decarboxylase (ODC). This enzyme converts ornithine into the diamine putrescine. If ODC is downregulated, then downstream higher polyamines would exhibit an overall decrease in response. Further experimentation is required in many aspects to confirm or refute these conclusions. More replicates are required to validate both alkaline phosphatase activity as well as quantification of mineralization. Second, measuring SAT1 mRNA and protein expression throughout osteogenesis to confirm continuous knockdown following transduction must also be performed. Third, HPLC would reveal polyamine levels following deficient SAT and whether they are in excess compared to control.

The insights gained from this project expand upon previous studies that showed variable results. Contradictory ideas stating polyamine supplementation assists in bone mineralization *in vitro*, while other studies suggest a lack of polyamines may improve bone growth. For example, spermine, spermidine, and putrescine supplementation have all been reported to increase mineralization (Facchini, 2012; Guidotti, 2013; Lee, 2013). On the other hand, DFMO, an ornithine decarboxylase (ODC) inhibitor that decreases subsequent higher polyamines has also been shown to support calcification of bone (Tsai,

2015). It has also been observed that SAT1 increases in response to both osteogenic and adipogenic growth media, suggesting that the acetylation and excretion of polyamines is important for those biological processes (Tsai, 2015). Inconsistencies in the literature might be present due to dissimilarities in cell type, cells derived from non-human species, and lack of biological replicates. Here we work with MSCs derived from healthy human donors where each biological replicate represents a different donor.

This project suggests that an excess of polyamines is inhibitory towards osteogenesis. It also demonstrates that SAT1 is significantly upregulated throughout osteogenesis, supporting the idea that excretion and lower levels of polyamines are necessary for proper bone mineralization. Restoring spermine synthase function through adeno-associated virus (AAV) delivery would be the most direct approach to providing a therapy for SRS patients. Beyond this, this work informs the idea that depletion of polyamines within affected tissues may prove to be a target for therapy. This could be done in a manner of ways, such as inhibition of ODC with DFMO, or overexpression of SAT1. Altogether, this work offers insight into the molecular mechanisms in which the osteoporotic phenotype within Snyder-Robinson Syndrome patients arises, and therefore may contribute to future therapeutics and drug development to improve quality of life in SRS patients.

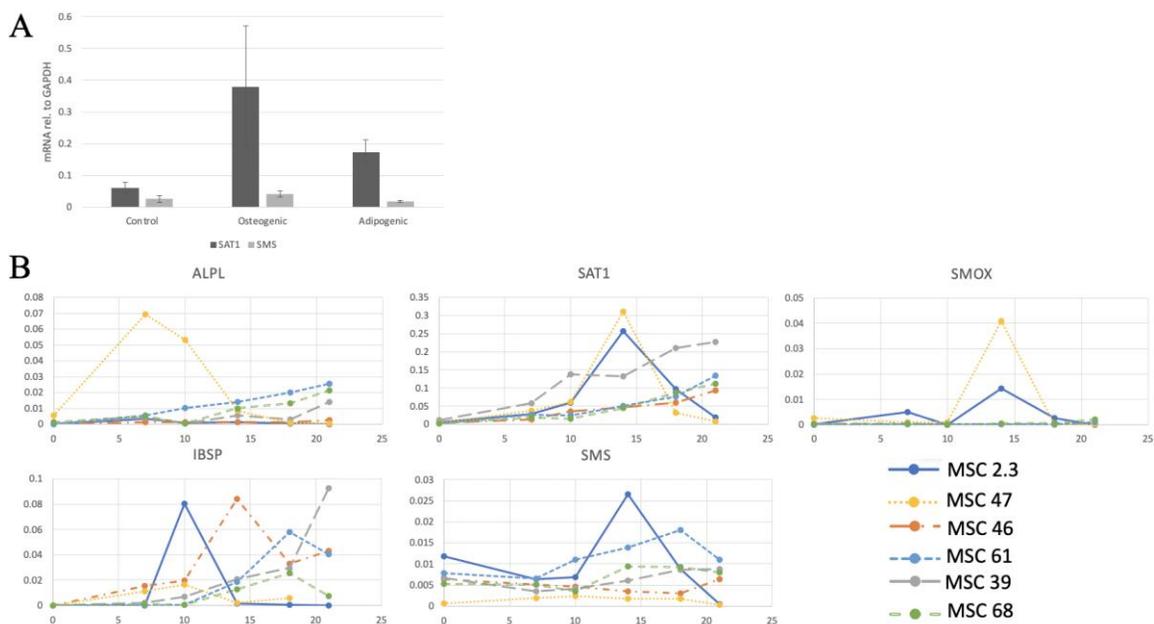
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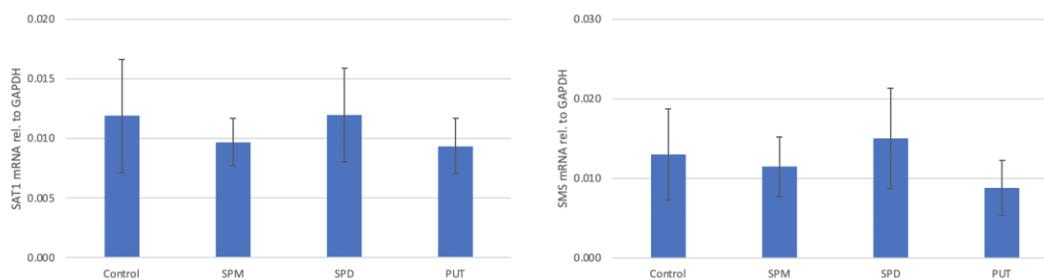
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Appendix A



Supplementary 1: Osteogenic differentiation greatly impacts SAT1 expression, however moderate trends within other polyamine pathway enzyme transcription. (A) Real Time PCR depicting mRNA levels of SAT1 and SMS (n=3). (B) Real Time PCR of different osteogenic markers and polyamine enzyme mRNA levels. Each line represents a biological replicate (n=6). Error bars represent standard error of mean. Statistical significance was determined using 1-way ANOVA, deeming changes are non-significant (n.s.). We expect that the upward trend in SAT1 (A) is n.s. due to the low number of replicates, and thus further biological replicates should be performed.

Appendix B**A**

Supplementary 2: Supplementation of exogenous polyamines has minimal effect on key polyamine pathway enzymes. Real Time PCR depicting mRNA levels of two key polyamine pathway enzymes in response to exogenous supplementation of polyamines at 10 μ M (n=6). These experiments were performed in standard MSC media. Error bars represent standard error of the mean. Statistical significance was determined by 1-way ANOVA, deeming changes in expression were non-significant (n.s.).