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Single Low Dose Bisphosphonate Treatment Enhances Osteogenesis in hMSCs By Exerting Epigenetic Changes

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ABSTRACT

Background: Bisphosphonates are analogues of pyrophosphate used to treat bone diseases, like osteoporosis and malignant bone diseases characterized by excessive bone resorption. It has been shown that bisphosphonates hinder bone resorption by interfering with osteoclast activity. **Objectives:** The present study is planed to investigated the extended effect of a single low dose of bisphosphonate on proliferative, osteogenic behaviour and if treatment with bisphosphonate leads to epigenetic changes in the human mesenchymal stem cells. **Methods:** We investigated the effects of a single low dose of two BPs [Alendronate (ALE) and Pamidronate (PAM)] on human mesenchymal stem cells (hMSCs) behaviour and phenotype. hMSCs were plated at a density of 5×10^5 cells. After 24 hr the medium was changed with growth media containing bisphosphonate at both 100nM and 10nM and were incubated for 24 more h. Cells were then washed, trypsinized and sub-cultured another time with no added exposure to drug. The cell cultures were assayed for proliferation and osteogenic differentiation as well as for changes in DNA methylation. **Results:** Treating cells with a single low dose of either ALE or PAM (100nM and 10nM) brings about a permanent change in the proliferative and osteogenic behaviour of hMSCs even after passaging the cells. **Conclusion:** The augmentation of osteogenesis points to the usage of low dose bisphosphonates as an adjunct to implant placement.

Key words: Bisphosphonates, Epigenetics, Mesenchymal stem cells, Osteogenesis, Osteoporosis.

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INTRODUCTION

Epigenetics means studying changes due to modification of gene expression rather than change in gene code itself. It is influenced by both internal as well as external triggers which result in DNA methylation. Studies have shown that altered DNA methylation affects behaviour of cells like human mesenchymal stem cells (hMSCs).[1,2] It has been shown that hMSCs retain memories of past environment that influence their behaviour. Gilberto *et al.* reported that the stem cells originating from muscle retain memory of the last mechanical stimuli, that might stay even following the *in vivo* application.[3] Surface topography of the substrate is also thought to influence the behaviour of hMSCs even at the nano scale level.[4] This effect may bring alterations in some of the epigenetic markers, that may afterwards promote tumorigenesis and be transmitted through the cell division cycle.[5,6]

Bisphosphonates (BPs) are a class of drugs that are used to treat resorptive bone diseases like osteoporosis.[7] They mainly inhibit osteoclast action by different ways. They also have effect on other cells like osteoblasts, keratinocytes, fibroblasts and stem cells.[8]

We carried this study to find out the effect of a single low dose of BPs on the proliferative and osteogenic behaviour of hMSCs by evaluating the extracellular matrix mineralisation parameters and to assess the ability of the hMSCs to synthesise osteoblastic markers. In addition we are looking for any concurrent epigenetic changes induced by performing a global DNA methylation screen and to find out whether these changes induced by exposure to BPs that cause phenotypic changes.

MATERIALS AND METHODS

Cell culture

Human Mesenchymal Stem Cells (hMSCs) were obtained from the Institute for Regenerative Medicine at the Texas University Health Science Center College of Medicine (USA). hMSCs were plated at a density of 5×10^5 cells/75cm^2 flasks containing MSC Growth Media (GM) containing 10% Fetal Bovine Serum (FCS) and 100U/ml each of penicillin/streptomycin. Cells were incubated at 37°C in humidified 5% CO_2. After 24 hr the medium was changed with GM containing Bisphosphonates Alendronate (ALE) and Pamidronate (PAM) at both 100nM and 10nM and the hMSCs were incubated for another 24 hr. Cells were washed, trypsinized and sub-cultured in GM without further exposure to BPs.

For the proliferation experiment 5000 cells/24 wells were used, while 10000 cells/24 wells were used in the other experiment. For the epigenetic experiment the hMSCs were plated at a density of 5×10^5 cells/75cm2 in flasks containing MSC GM. After 24 hr, the medium was changed to use GM containing BPs at both 100nM and 10nM and the hMSCs were incubated for a further 24 hr. The cells were then washed, the medium was changed and the cells were grown for a further 2 weeks, during which time the medium was changed twice a week. On reaching this stage, the cells were then ready for DNA extraction and to assess the epigenetic changes.

Proliferation

Proliferation was measured using an Alamar blue assay. A 10% dye solution was added to each well and cells were incubated for 4 h at 37°C

and 5% CO_2. Next, the fluorescence intensity (Excitation: λ = 530 nm, emission: λ = 590 nm) was measured using a plate reader (BioTeK FLX800). The total cell numbers were calculated via interpolation through the use of a known standard curve.

Von Kossa staining

To semi-quantify the mineralisation produced by cells, such as osteoblasts, Von Kossa staining had been used according to manufacturer's instructions (Abcam, UK). The samples were stained and then examined using an Olympus BX50 optical microscope (Olympus, Southall, Middlesex, UK) along with a Cool snap PRO-cf and camera (Media Cybernetics, Marlow, Buckinghamshire, UK). Images were processed to semi-quantify the calcium crystals using Image-Pro Plus v4.5 (Media Cybernetics, Marlow, Buckinghamshire, UK) - software.

Hydroxyapatite

To measure the total amounts of hydroxyapatite (HA) formed by hMSCs; OsteoImage mineralisation assay (Lonza, UK) was used according to the manufacturer's instructions. By the end of the experiment, the culture media was removed and the cells were then washed twice in PBS then fixed in 4% paraformaldehyde for 15 min. Fixative agent was discarded followed by twice washes with diluted wash buffer. Following this, the cells were incubated with staining reagent in the dark for 30 min at room temperature followed by several washes with a diluted wash buffer. After that, we added an appropriate amount of wash buffer to the wells for microscopy viewing and plate reader analysis. Visual examination was carried out using a Leica–DMIRB fluorescence microscope equipped with COOLSNAP Monochrome Camera. Images were collected and processed with the Image J Imaging System.

Epigenetic and DNA methylation

DNA from all cultures was extracted using a QIAamp DNA Mini kit from Qiagen according to the manufacturer's instructions. This step followed bisulphite conversion by taking a 500ng of genomic DNA using DNA Methylation™ Kit (Zymo Research, USA) according to the manufacturer's instructions. Finally, the Human Methylation 450 bead chip array processing was conducted according to the Infinium HD Assay protocol (15019519_B, 2015) (Illumina Inc, San Diego, USA). Data were normalised using the functional normalisation function within the Minfi Bioconductor package and then further pre-processed using the Minfi functions 'detP' and 'getSnpInfo' to filter out probes not meeting a detection p-value threshold of 0.01or probes near a SNP.[9,10] Cross-reactive probes were also filtered out by matching to the list published by Chen *et al.* 2013. The normalised and filtered data was analysed for differential CpG probes using Limma.[11] M-values were used in the regression. To avoid infinite M-values, beta values of exactly 0 or 1 where shifted to the value of 1×10^{-4} using the shift Beta function in Harman and these adjusted beta values were logit transformed to M-values.[12]

For the limma differential methylated probe analysis, a simple linear model of y ~ 0 + x was used. There were no experimental covariates and the gender of cellular origin was the same for all samples so a more complex model did not have to be specified. To determine differential methylation, contrasts were made of the ALE and PAM treated samples against samples in the GM. Contrasts were made for both the 100 nM and 10 nM concentrations of ALE and PAM against the GM. As well as differential tests for CpG sites, detection of Differentially Methylated Ranges (DMRs) was also undertaken using DMRcate. Gene ontology and KEGG pathway analysis was undertaken using the 'gometh' function in the missMethyl Bioconductor package.

Statistical analysis

hMSCs from three donors (N=3) were used in triplicate (n=3). Statistical analysis was performed by one-way Analysis of Variance (ANOVA) in GraphPad Prism software (v5.04) followed by Bonferroni post-test; the representative data is presented as mean ± Standard Deviation (SD), with $p<0.05$ considered to be statistically significant. The DNA methylation data analysis was carried out using R software version 3.3.2 and Bioconductor Illumina Human Methylation 450 packages. To detect the significant hypermethylated and hypomethylated genes-associated probes, the limma decide tests function was performed to classify a series of related t-statistics as up, down or not significant. The default was Benjamini–Hochberg-false discovery rate adjusted p values ($p<0.05$).

RESULTS

Proliferation

The proliferation of 5000 cells/24 wells of hMSCs in GM was examined at one, three and seven days. ALE (100nM and 10nM) stimulated significant cell proliferation in all cells on Day 3 and Day 7. Also, cells that had been treated with 10nM PAM on Day 3 and Day 7 showed significant proliferation when compared to control cells that were treated with GM only. Cells treated with 100nM PAM promoted cell proliferation, but this increase was not statistically significant. Both drugs have shown a similar effect on cell proliferation at Day 1 (Figure 1).

Calcium deposition assay

Calcium deposition believed to be one of the markers that linked to late stage osteogenesis process. Our results suggest that treating cells with a single low dose of drugs (100nM and 10nM) can bring out permanent changes in the osteogenic behaviour of the hMSCs even after passaging the cells. Data showed that both PAM (100nM and 10nM) drug significantly stimulated calcium deposition after three weeks when compared to cells treated with osteogenic media only. However, for the group treated with ALE (100nM and 10nM) there were more calcium deposition but it was not a statistically significant.

Von Kossa Staining

In order to visualise the mineralised nodules following single low dose treatment of BPs a von Kossa stain was used. Visual inspection showed that there was a significant effect on mineralized nodule formation. All groups treated with ALE and PAM (100nM and 10nM) were positive to Von Kossa stain in comparison to the control group that been treated with OM only (Figure 2).

Collagen deposition

A Sircol collagen assay kit from Biocolor was used to analyse the collagen deposition following the manufacturer's instructions. The data showed that both ALE and PAM at both concentrations significantly stimulated hMSCs to produce more collagen at 14 days following a single low dose of drugs (100nM and 10nM) than the other group that was with osteogenic media.

Hydroxyapatite

Hydroxyapatite nodule formation was visually examined microscopically (Leica DMRIB Fluorescence microscope). Data showed that after 21 days of culture in OM media following single dose treatment either with ALN or PAM there was a clear difference in the appearance and formation of the hydroxyapatite nodules when compared to the control group that had been treated with OM only. HA depositions were semi-quantified using Image Pro Plus software, version 4.5 to calculate the percentage

of fluorescent area per 100μm^2 and the results correlated with visual inspection (Figure 3).

Alkaline phosphates activity

ALP enzymes are one the most useful early osteogenic markers. The level of ALP activity was studied after seven days of incubation in OM. The data showed that the groups treated with a low dose of ALE and PAM (100nM and 10nM) significantly stimulated the ALP activity when compared to the control group that treated with OM only.

Epigenetic and DNA Methylation

In all, 89,647 probes failing one of more filters were discarded. For a genome-wide overview of the DNA methylation changes due to the drug, it is useful to perform Multidimensional Scaling (MDS) using the Euclidean distance of each sample from one another (Figure 4). To limit the computational overhead, M-values of the 100,000 most variable CpG probes were used in the dimensionality reduction. The first MDS dimension had a correlation with the number of failed probes (numbers below the sample name), this suggests the largest source of variation in the data is technical and not biological. It can also be observed on the second dimension projection that cells on either of the PAM media were the most epigenetically different to cells on GM. This suggests PAM supplementation has a larger effect on the methylome than ALE. The PAM replicates also cluster closely within the group, while the ALE replicates were more variable, partially due to having a replicate with high probe failure rate. There was also relatively high variance amongst the GM replicates. One of the GM replicates had a similar methylome to the ALE and OM samples.

Differentially Methylated probes (DMPs)

The most significant probe for the group treated with 100nM PAM was cg22867714, which is a promoter of the Von Willebrand factor gene. For the group treated with 10nM PAM, cg20733436 was the most significant probe and is responsible for the TMED7-TICAM2 gene.

Differential Methylation Regions

We found 386 DMRs for the PAM 100nM and 515 DMRs for the PAM 10 mM comparisons. Matching across the two sets of DMRs showed 210 regions had overlapping genome coordinates. In order to identify the most detectable CpGs, the average percentage change of DMR values was calculated, βs, after normalisation by best and worst performing samples.

DISCUSSION

Literature revealed that stem cells, in conjunction with biomaterial scaffold, were used to engineer various tissues and later in repair of tissue defects. The materials comprise collagen, agarose, Polymethyl Methacrylate (PMMA) and fibrin.[13] The scaffold provides an environment for stem cells to proliferate and differentiate and cell response varies depending on the substrates type. Various signalling molecules are involved in stem cell differentiation, like Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Transforming Growth Factor beta (TGF-β) and Wnt signalling family.[14] In our study we have shown that BPs, when used as external triggers, could be an additional factor to be manipulated for regenerative purposes.

In this study we have found that ALE and PAM have an extended effect on and accelerate hMSC proliferation on a plastic tissue culture substrate. Still it is unclear whether this effect may involve cell signalling pathways like. Despite hMSCs are considered as one of the most sensitive cells to external chemical and mechanical stimuli, our data supports these findings with even more permanent effects.[12-14]

The ability of hMSCs to differentiate to osteoblasts is a crucial factor for bone formation and repair.[15] ALP is one of the early markers of osteogenesis which is involved in the calcification of the bone matrix. Both the drugs were reported to stimulate ALP activity at Day 7. Thus showing that the drug has an early impact on osteogenic differentiation and the production of extracellular minerals. Furthermore both ALE and PAM promotes considerably higher level of calcium deposition which is established by qualitative and quantitative approaches.

We used von Kossa stain to confirm the presence of physiological mineralization nodules. We found a significantly increased bone mineral level when HA parameter was used. HA is mainly used in bone graft substitutes or in implant coating so as to promote bone formation around the implant and to restore the missing bone.[16] In our study we found that low dose BPs stimulate hMSCs to produce more HA minerals, which might enhance the bone to implant contact, increase the bone apposition rates and accelerate bone healing. Usually the osteogenic differentiation process takes up to four weeks. We found in our study that the drugs may even shorten this time period to three weeks. This finding is similar to study by Ge *et al* who also reported that external stimuli up regulate and accelerate the osteogenic differentiation process.[17]

Earlier studies showed that collagen deposition in the extracellular matrix is a vital factor in the bone micro-environment that contributes in the haematopoiesis process, healing and the bone remodelling. We found that both drugs have a stimulatory effect on extracellular collagen type I, which may play an integral role in bone turnover, especially with bone related problems. Therefore, treating hMSCs with a low dose of BPs may be involved in the maintenance and growth of bone. Furthermore, from these findings we wanted to assess the ability of the extended effect of this drug in controlling and regulating the initiation of hMSCs' osteogenic behaviour on wound healing, as these cells are considered the first cells recruited to sites of bone trauma and around implant surface.[18]

Several factors might impair the bone formation and remodelling like age, diabetes mellitus, osteoporosis, smoking and alcohol abuse. Both *in vitro* and *in vivo* studies have reported that bone marrow stem cells will augment fracture repair and consequently accelerate wound healing. Hernigou *et al.* reported that around 88% of tibial non-unions were treated successfully after application of autologous bone marrow MSCs on a tibial non-union fractures.[18,19] We suggest that hMSCs in combination with drugs may be used as a promising approach to repair bone defects and also in healing wounds. Our finding presents a way for promising systematic approach to accelerate the osseointegration and increase bone-implant contact without coating implants.

Our study has shown that the BPs may direct the stem cell differentiation behaviour towards one lineage. As stem cell differentiation process is a complex one and is mediated by several internal and external factors, it is still difficult to decide at what stage or which factor is involved in this effect. However, as the nature of the changes being permanent suggests a probable epigenetic mechanism in force. We showed that the low dose of BPs was associated with epigenetics changes in hMSCs via DNA methylation mechanisms. The dependency of the effect of DNA methylation on BP dose and type was expected. It was shown that both the 100 nM and 10nM PAM doses had an effect on hMSC phenotype and behaviour. This effect might be due to a cellular interaction with external stimuli like chemical stimuli. In addition, our data suggest that the low dose of PAM may have acted as an external trigger that may have committed the cells to differentiate to a specific lineage, as well as increasing their proliferation rate and accelerating osteogenic differentiation. In addition, our study also showed that the low doses of 100nM and 10nM PAM significantly affected a wide range of gene probes. As each gene has a distinctive role in cellular development, as well as proliferation and differentiation.

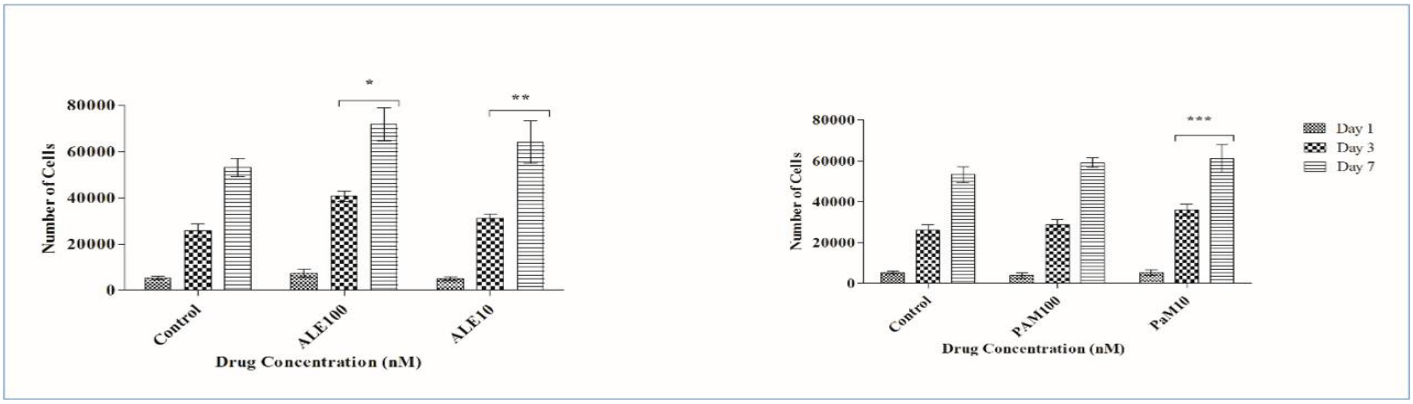

Figure 1: The effect of the ALE (100nM and 10nM) significantly stimulated cells proliferation at Day 3 and Day 7.

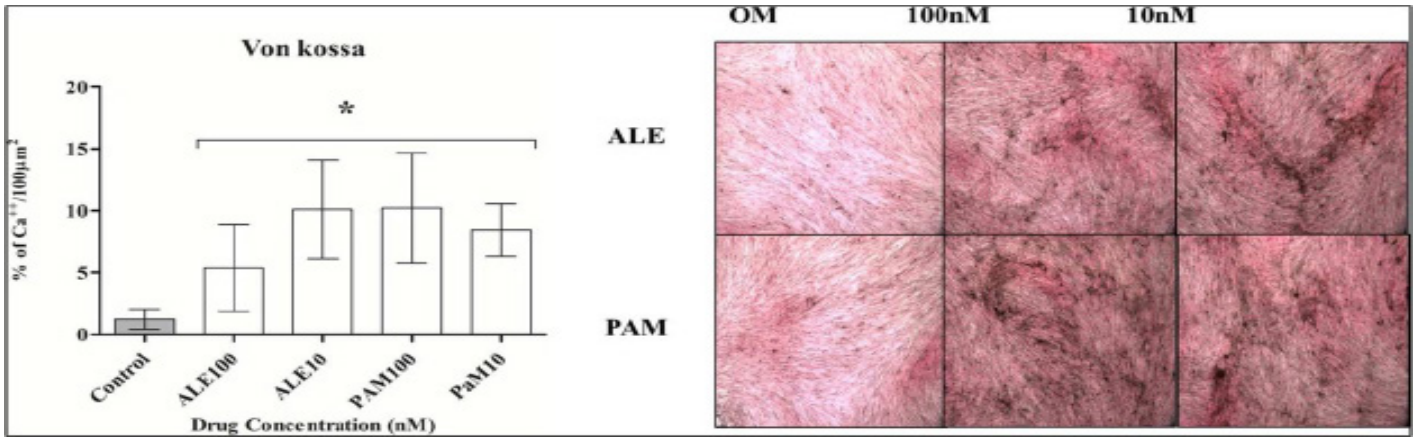

Figure 2: More mineralized nodules (black area) produced by hMSCs osteogenesis after 21 days in culture than the group treated with OM media only.

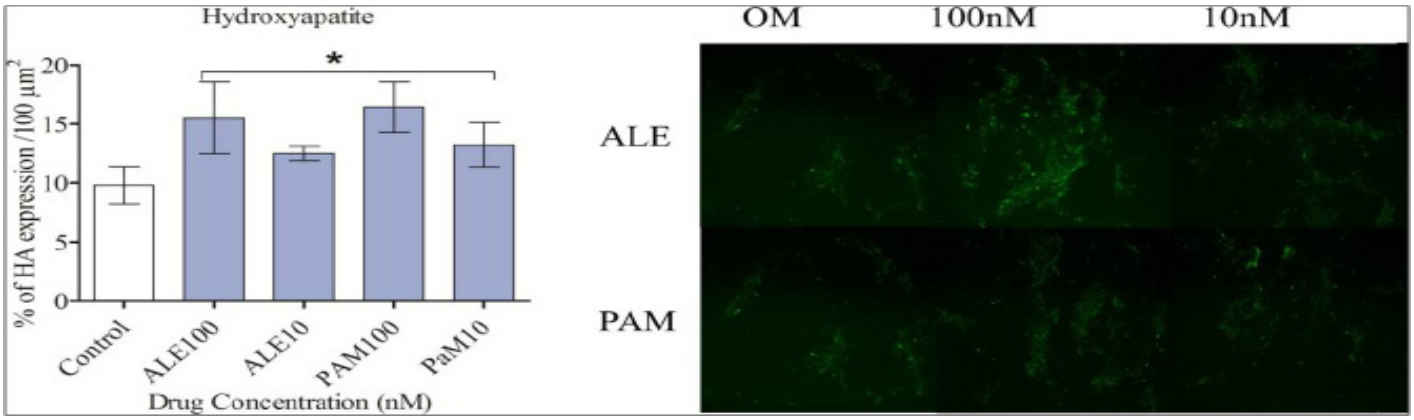

Figure 3: Florescent microscopy images showed more deposited hydroxyapatite was produced by hMSCs than the group treated with OM media only.

The exact role and involvement of various genes in the life, growth and development of cells, chiefly stem cells, remains to be explicated.

A number of DMPs were reported to be associated with the bone formation or remodelling. Mukherjee *et al.* showed that Akt promotes BMP2-mediated osteoblast differentiation and bone development. While similar studies showed that Carbonic Anhydrase 4 (CA4) functions in bone calcification and prolyl 4-hydroxylase (P4HA2), enzyme is involved in collagen synthesis. Other DMPs were also found to be associated with genes involved in hMSC homing or inflammation like von Willebrand factor (VWF) for endothelial cell adhesiveness and Coagulation Factor XII (F12) and tissue-type plasmogen activator, or tPA (PLAT) involved in blood clotting. It has also been reported that two genes, Nitric Oxide Synthase Interacting Protein (NOSIP) and Nitric Oxide Synthase 2 (NOS2) are involved in nitric oxide signalling in inflammatory processes.[20,21]

The current findings have some limitations, such as the fact that the drug effects were studied in an *in vitro* environment and other factors may be involved in these mechanisms as in the *in vivo* environment. Therefore,

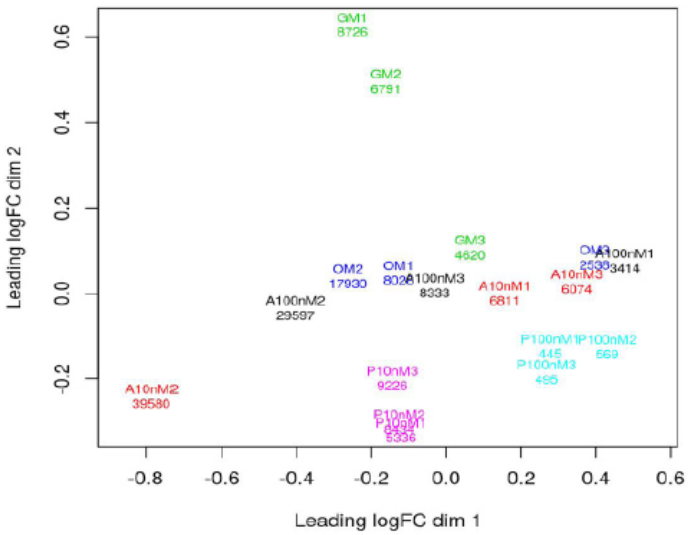

Figure 4: Classical multidimensional scaling (MDS) plot of the 100,000 most variable probes.

the *in vitro* approach is considered as a useful measure for early screening of potential targets and also provides an early step in testing drug effects and possible implications. However, in an attempt to overcome this limitation, we examined at least three methylation sites in CpG for each gene regulatory region. All of the cells used in this project came from male donors and there was little evidence of a gender effect on phenotypic changes. Furthermore, epigenetic changes may be influenced by the source of hMSCs, whereby the cells may have already undergone initial alterations in DNA methylation, but to the best of our knowledge no clear evidence of that has previously been shown.

CONCLUSION

The outcome of this study has shown that the BPs enhanced both early and late osteogenic markers. Also, there was an extended effect on hMSCs' proliferation behaviour. They indicate these drugs have a strong effect on cells in depositing calcified matrix and producing more collagen. These finding suggest that an understanding of the phenotypic changes that occur during the life cycle of hMSCs could provide a clear vision with regard to the success of using these cells in the medical field and an understanding the underlying mechanisms of these external triggers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ALP: Alkaline phosphates; **ALE:** Alendronate; **BPs:** Bisphosphonates; **hMSCs:** Human mesenchymal stem cells; **GM:** Growth medium; **OM:** Osteogenic medium; **PAM:** Pamidronate.

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