Identification of novel epigenetic pathways regulating cranial mesenchymal stromal cell fate determination.

A Thesis submitted in partial fulfilment of the

HONOURS DEGREE of

BACHELOR OF HEALTH AND MEDICAL SCIENCES in

The Discipline of Physiology

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By Alexander Yeo

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Abstract

Introduction: Craniosynostosis is characterised by the premature fusion of cranial sutures affecting approximately 1 in every 2,500 live births. Premature fusion of the sutures leads to abnormal skull development and increased risk of cognitive deficits due to elevated intracranial pressure on the growing brain. Saethre-Chotzen syndrome (SCS) is a common form of craniosynostosis caused by a loss of function mutation in the TWIST-1 gene, resulting in dysregulated osteogenic progenitor cells. Current treatment of craniosynostosis involves multiple traumatic, potentially life-threatening surgical interventions to remove the excess bone from the top of the skull, emphasizing the need for therapeutic interventions. Our lab has previously reported on the epigenetic control of osteogenesis via histone/DNA methylation and demethylation. The aim of this study was to develop an osteogenic based reporter cell line for high-throughput screening of a chemical epigenetic library.

Method: The initial work involved infecting different cell populations that have different osteogenic potential with lentiviral dual promoter fluorescence reporter (LVDP) constructs driven by osteogenic regulator promoters, Collagen1α1, VitaminD_3 responsive element and Runx2. Cells were infected with different LVDP constructs and the assessed in real-time under osteogenic conditions for up to 14 days.

Conclusions: Analysis of fluorescence levels of infected cells identified the Runx2 reporter as a possible candidate for the screening of the chemical library. It is hypothesised that the chemical screen will allow us to uncover novel epigenetic regulators and inhibitors of osteogenesis. This is fundamental in the development of an effective, reversible treatment for craniosynostosis in SCS patients.

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Introduction

Cranial Development

Normal human cranial development is dependent on the time-appropriate fusion of the bony plates at calvarial sutures, which enclose the brain. Primary ossification centres are established during embryogenesis, leading to suture formation. Bone grows radially from these centres mid-gestation, resulting in the formation of the metopic, coronal, sagittal and lambdoid calvarial sutures. Previous studies have established the presence of a reservoir of Gli1+/Twist-1+ cranial stem cells residing in the suture mesenchymal stem cells (MSC)[1,2,3]. Osteogenic progenitor cells arise from this reservoir, and differentiate into mature osteoblasts responsible for bone formation. Following extracellular matrix mineralisation, terminal differentiation into osteocytes occurs completing the osteogenic differentiation lineage (Fig 1).

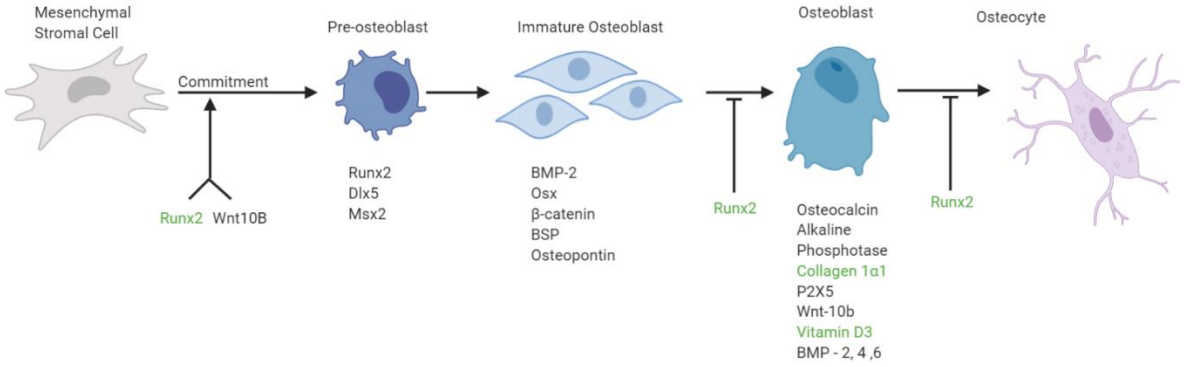


Figure 1. Osteogenic markers involved in MSC osteogenic lineage commitment. Genes and pathways associated with osteogenic differentiation are highlighted above. Runx2 is a master regulator of osteogenesis involved multiple phases of MSC differentiation. Additionally, Collagen1α1 and VitaminD_3 response element transcription factors play large roles in osteoblastic differentiation.

The sutures, excluding the metopic suture, remain unfused until early adulthood[4,5]. Craniosynostosis is a medical condition characterised by the premature fusion of these sutures leading to increased risk of cognitive deficits due to elevated intracranial pressure as potential

cranial volume is stunted. Saethre-Chotzen syndrome (SCS) is defined as a loss of function mutation in the TWIST-1 gene, resulting in dysregulated proliferation of progenitor cells at the suture mesenchyme with consequential suture fusion.

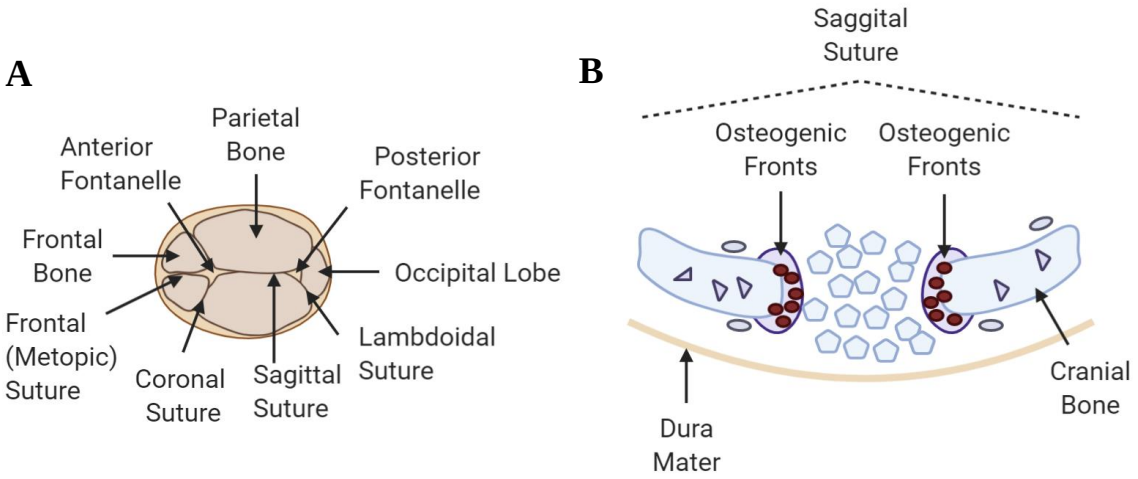


Figure 2. Graphic representation of the cranium (A) and sagittal suture (B). Existing cells present in the suture and surrounding bones are *osteoblasts (●) pre-osteoblasts (0), osteoprogenitors (⬟), osteocytes (▲)*[1].

SCS is characterised by fusion of the coronal suture (Fig. 2) and patients exhibit craniofacial and limb deformations. Dysregulated osteoblastic proliferation and subsequent differentiation impacts both intramembranous and endochondral ossification, leading to craniosynostosis[6,7]. ***Thus, calvaria defects and cranial bone anomalies are linked to uncontrolled intramembranous and endochondral ossification. Furthermore, research shows that Twist-1 is a key gene involved in nominal MSC growth, proliferation and differentiation***. TWIST-1 encodes a basic helix-loop-helix transcription factor, critical in the control of healthy skeletal development[8,9,10]. Our laboratory has demonstrated that TWIST-1 expression is high in the most immature human bone marrow-derived mesenchymal stem cells (BMSC)[11], a population of non-haematopoietic progenitors that give rise to the osteogenic lineage[12]. Additionally, previous research has shown that cranial parietal cells from SCS patients express decreased levels of TWIST-1 in contrast to the levels expressed by normal control cells[13]. In addition,

SCS derived cranial cells have been shown to have high capacity for mineralisation under osteogenic conditions and limited life-span *in vitro*[14]. Our group has also demonstrated inhibition in osteogenic differentiation of TWIST-1 haploinsufficient calvarial cells in SCS patients through the inhibition of a TWIST-1 target gene, *Tyrosine kinase receptor c-ros-oncogene 1 (C-ROS-1)*[15], which is normally supressed by TWIST-1. ***These findings reflect the relationship between Twist-1 heterozygosity and cranial/limb abnormalities***[16] ***and is indicative of the regulatory function of Twist-1 in BMSC and MSC regulation, with limited knowledge of downstream Twist-1 mechanisms involved in SCS suture fusion***.

Epigenetic Gene Regulation

Chromatin histone modification plays a key role in gene transcription and lineage determination[17].

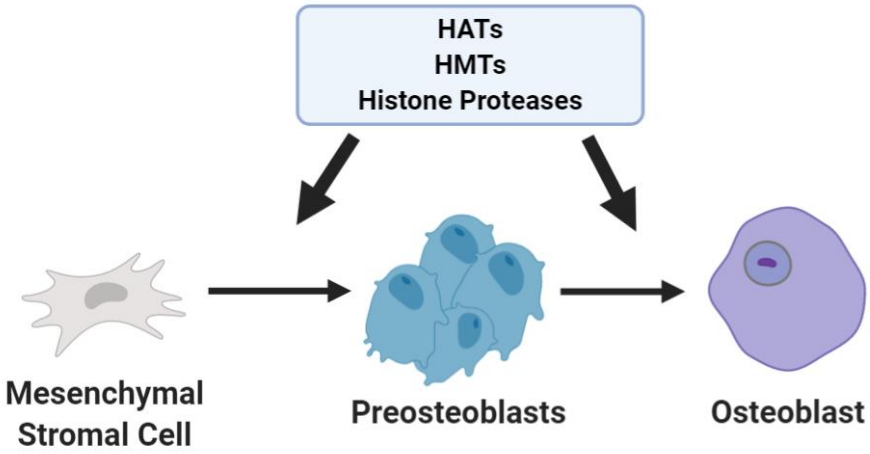


Figure 3. Histone/lysine acetyltransferases (HATs), histone methyltransferases (HMTs) and histone proteases on osteogenic lineage. Histone modifying enzymes alter the transcription of genes. HATs serve as transcriptional coactivators and catalyse lysine acetylation leading to loosening of chromatin and subsequent transcription of genes[18].

These modifications are vast and complex, with little known about specific pathways and mechanisms of action[19]. These modifications control gene expression by modifying chromatin

structure and availability of transcription factors to promoters and enhancers without altering the DNA sequence itself[20], some of which are represented in Figure 3. This is known as epigenetic regulation[21]. Published reports suggest that epigenetic modifications play a role in MSC osteogenic differentiation as well as multiple lineage transdifferentiation, ***however exact mechanisms for the latter are largely unknown***[22].

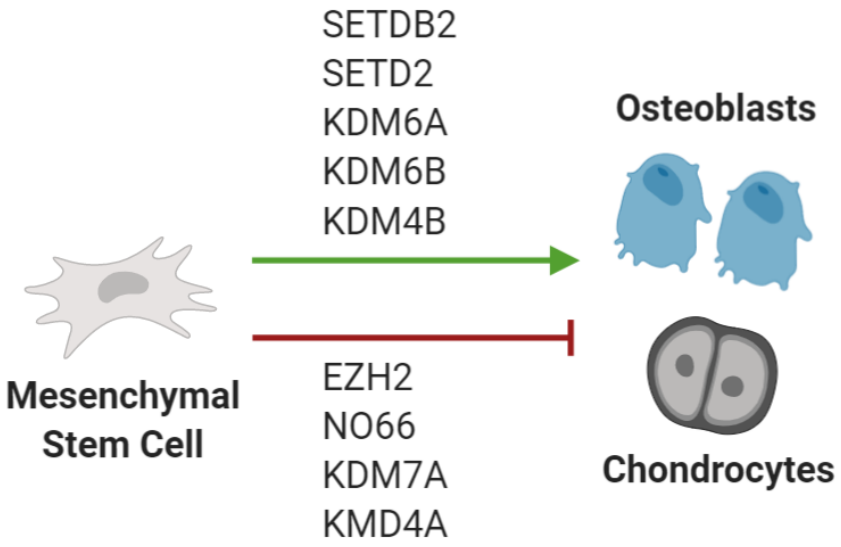


Figure 4. Known epigenetic enzymes regulating MSC lineage commitment. Known epigenetic enzymes (histone methyltransferases/demethylases) acting as mediators of stem cell differentiation[25].

The epigenetic modifier EZH2 tri-methylates histone H3 on lysine 27 (H3K27me3), recruiting DNA methyltransferases for gene silencing[23]. Our lab has shown that enforced expression of EZH2 in BMSC represses transcription of osteogenic genes via the induction of H3K27me3, where conditional knockout of EZH2 results in craniosynostosis[16,17]. ***Based on this, H2K27me3 regulation is a likely critical factor involved in MSC lineage commitment and nominal cranial suture development***. Our studies have also implicated H3K27 demethylase KDM6A as a counterpart to EZH2 activity to regulate BMSC (Fig. 4)[24].

Roles of Runx2, Collagen1α1, and VitaminD$_3$ in the osteogenic lineage commitment

For the purposes of this study, runt-related transcription factor 2 (Runx2), Collagen1α1 and VitaminD$_3$ responsive element were selected as they are key regulators of osteogenesis. A master transcription factor, Runx2 is critical for osteoblastic differentiation. Expression of Runx2 in the cranium is essential for cranial suture closure[26]. Runx2 mutations lead to cranial defects[25,24]. Runx2 effectuates expression of early osteoblast differentiation markers Osteocalcin, Osteopontin and COL1α1[27]. Collagen 1 is a protein encoded by the COLA1α1 gene and is an osteogenic marker associated with osteoblastic differentiation[28]. Studies have shown that osteoblasts must be in contact with a Collagen I type 1 matrix so they can begin differentiation[27,28,29]. Therefore, Collagen1α1 is a vital proponent of osteogenesis and was as such selected as an appropriate osteogenic marker to assess for the purposes of this study. VitaminD$_3$ also plays a large role in osteogenesis, with studies reporting FGF-R blocking results in decreased activity of VitaminD$_3$ transcription factors[30]. VitaminD$_3$ acts primarily through the actions of its active metabolite 1,25 dihydroxycholecalciferol (1,25(OH)2D3/Calcitriol). Having been enzymatically produced by VitaminD$_3$, 1,25(OH)2D3 binds to a Vitamin D Receptor (VDR), a nuclear receptor within cells[30,31]. Osteoblasts are one such cell that expresses VDR and is thus directly differentially affected by the action of 1,25(OH)2D3[32]. VitaminD$_3$ influences both bone formation and mineralization, as well as acting to prevent excessive bone deposition[33.] Therefore, the LVDP VitaminD$_3$-RE was selected as a potential candidate reporter construct to monitor osteogenesis.

Gap in Knowledge

Utilizing novel epigenetic factors influencing aberrant MSC osteoblastic differentiation to develop a potential drug-based treatment option for syndromic craniosynostosis is fundamental in replacing traumatic, high-risk correctional procedures. Current treatment options for

craniosynostosis rely on invasive cranial surgery to reshape and reposition ill-forming bones allowing volume for brain growth. Excision of excess suture tissue is highly traumatic, carries many inherent risk factors and often requires multiple surgeries to be an effective. Therefore, in order to greatly reduce the risk to patients, an effective non-surgical treatment should be explored. Epigenetic changes in gene expression occur independent of DNA sequence and are therefore potential targets of reversible drug treatments. Despite research yielding the discoveries of epigenetic modulators such as KDM6A and B, there is a gap in knowledge regarding epigenetic deregulation in SCS where no current epigenetic based drug therapy exists. Development of a non-invasive therapy that epigenetically inhibits osteogenic fusion of the sutures could be greatly advantageous for both patient recovery and quality of life. Therefore, the project will revolve around uncovering novel epigenetic regulators of osteogenesis in cranial MSCs with the goal of developing a potential drug-based therapy to treat premature suture fusion.

Hypothesis and Aims

Hypothesis: It is hypothesised that novel epigenetic regulators of osteogenesis can be identified in a chemical library by screening with stromal cells transduced with viral fluorescence reporter constructs driven by osteogenic promoters.

Aims:

1) *To identify the most suitable lentiviral osteogenic fluorescence reporter by infecting selected cells/cell line and examining their response under osteogenic conditions.*
2) *Undertake a screen of a chemical library (Cell Screen SA) using selected reporter lines based on osteogenic promoters.*
3) *Confirmation of the effectiveness of discovered inhibitors on human cranial cells.*

Materials & Methods

Lentiviral Dual-Promoter Constructs

Three *Lentiviral Dual-Promoter (LVDP)* reporter constructs were generously supplied by Stelios Andreadis, Ph.D., of the University of Buffalo (Fig 5). These LVDP constructs contain two independent promoters driving expression of two separate reporters for the purposes of firstly identifying viral infected cells and secondly, assessing osteogenic gene activation in real-time (Fig. 5). The viral reporters for Runx2 and Col1a1 contain a gene promoter (P), and the VitaminD_3 reporter contains a transcription factor binding site (Response Element (RE)) driving the expression of a ZsGreen reporter protein (GFP), indicative of osteogenic pathway activation. A second CMV or PGK promoter constituently drives expression of a discosoma red reporter protein (RFP) as a tool to measure viral data internalization[34]. LVDP constructs carrying a Runx2-P, Collagen1α1-P, and a VitaminD_3-RE were selected as appropriate candidates to screen a chemical library to identify potential epigenetic inhibitors of osteogenesis.

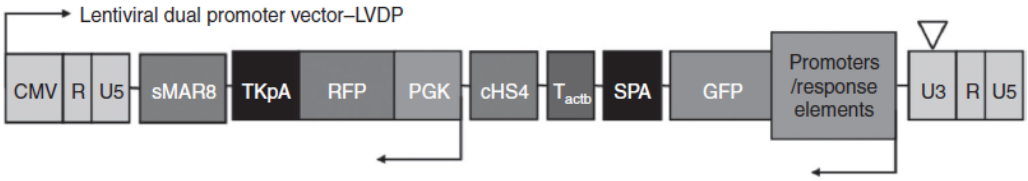


Figure 5. LVDP Construct Schematic. A schematic as produced by the LVDP developers illustrating the structure and basic function of the vectors[34].

Transformation and Purification

Plasmid DNA is incorporated into a cell through the process of bacterial transformation, for the purpose of utilising bacterial plasmid production and a means of generating replicates of desired plasmids (Fig 5). In this context, E. *coli* strain JM109 was transformed with the 3 viral reporters and a packaging vector, pMD2.G individually. All bacterial work was conducted in

the presence of a flame so as to maintain sterility throughout the process. 2µl (50ng) of DNA was mixed with 50µl of competent cells and incubated on ice for 30 minutes. Each transformation was subsequently heat-shocked on a 42°C heat block for 90 seconds, thereby allowing supercoiled DNA to enter the cell via the disrupted cell membrane. Competent cells were then immediately placed back on ice for 3 minutes to ensure plasmid retention. 1ml Luria broth (LB) was added and cells incubated at $37^{O}C$ for one hour for ampicillin resistance gene to be expressed. Each transformation was streaked onto an (100µg/ml) Ampicillin-LB agar plates, and single colonies grown were incubated in 200ml of LB with 2ml of ampicillin (100µg/ml diluted from a 100mg/ml stock of ampicillin) to prevent unwanted growth of foreign contaminants. These batches of transformed competent cells were then used for DNA midipreps as per Nuceobond Xtra Midi Plus DNA purification kit protocol (catalogue no. NC1270087).

Cell culture

Human embryonic kidney 293T (HEK293T) cells were used as target transfection cells for the purpose of making viable virus due to their high degree of transfectability. HEK293T cells were cultured in high-glucose DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% transfection-grade fetal calf serum (FCS; HyClone, Batch no. KPJ22093) and additives containing: 200 mM L-Glutamine (SAFC Biosciences), 100 mM sodium pyruvate (Sigma Aldrich), 50 U/mL penicillin, 50 µg/mL streptomycin and 1 M HEPES buffer solution. Infection target cells including bone marrow stromal cells (BMSC), craniosynostosis bone cell line, gingival fibroblasts and Saos2 (osteosarcoma) were cultured in alpha-modification minimum essential medium (MEM), with 10% FCS and 4.5% of aforementioned additives. Osteogenesis was induced in target cells through the introduction of osteogenic differentiation media, containing; 5% Osteo-FCS, additives, 0.1 µM dexamethasone, 2.64 mM potassium phosphate (K_3PO_4) and 100 µM L-ascorbic acid. All cells cultured in 5% CO_2 at 37°C.

Transfection of HEK293T cells and infection of target cells with LVDP viral particles

HEK293T cells were seeded into 9x6cm dishes ($2x10^6$ cells/dish) and cultured in 3ml of transfection media (TF). The following day, media aspirated and 2mL of fresh TF media added to the dishes. A Lipofectamine 3000 mixture made by added 45µL Lipofectamine 3000 dropwise to 1500µL of serum-free DMEM (3 reactions) and incubated at RT for 5 minutes. A second mixture containing 18.36µL (653.7µg/mL) pMD2.G envelope plasmid, 16.36µL (917µg/mL) psPAX2 packaging plasmid 39µL P3000 enhancer reagent and 12.22µL (1227µg/mL) purified Vitamin D_3 plasmid DNA was created. The Lipofectamine 3000 mixture was added dropwise to the second solution and incubated at RT for 30 minutes. 1mL of the total solution was added dropwise to the HEK293T dishes, left to incubate for 2 days. This process was repeated for each P/RE. RFP expression indicative of transfection efficiency was visually assessed 2 days following using a fluorescence microscope. Target cells as mentioned were seeded in 2x6-well plates ($1.0x10^5$ cells/well) per reporter construct, and collected viral supernatants was filtered through 0.45µm low-protein binding filters to be added to each respectively labelled target cell plate. 1µL/mL of polybrene was added to the viral supernatant to enhance the efficiency of viral infection. Infection protocol was repeated the following day.

Fluorescence-activated cytometric sorting (FACS) of infected cells and induction of osteogenesis

FACS isolated RFP+/GFP- cells were seeded in 24-well plates (9,400 cells/well). Upon confluency, osteogenic differentiation media (DM) and normal growth media (GM) was added to the appropriate wells with bi-weekly media changes throughout the duration of the experiment. Fluorescence images were taken over a 14-day time course at 0 days, 3 days, 7 days and 14 days following osteogenic induction. Cells treated with differentiation media was first digested with Collagenase/Dispase, followed by liberation of adhered cells via trypsin

treatment. Samples were taken at +3 days, +7 days and +14 days for flow cytometric analysis for fluorescence quantification and intended statistical analysis.

Results

Infected cells were initially RFP+/GFP+ expressive before osteogenic induction

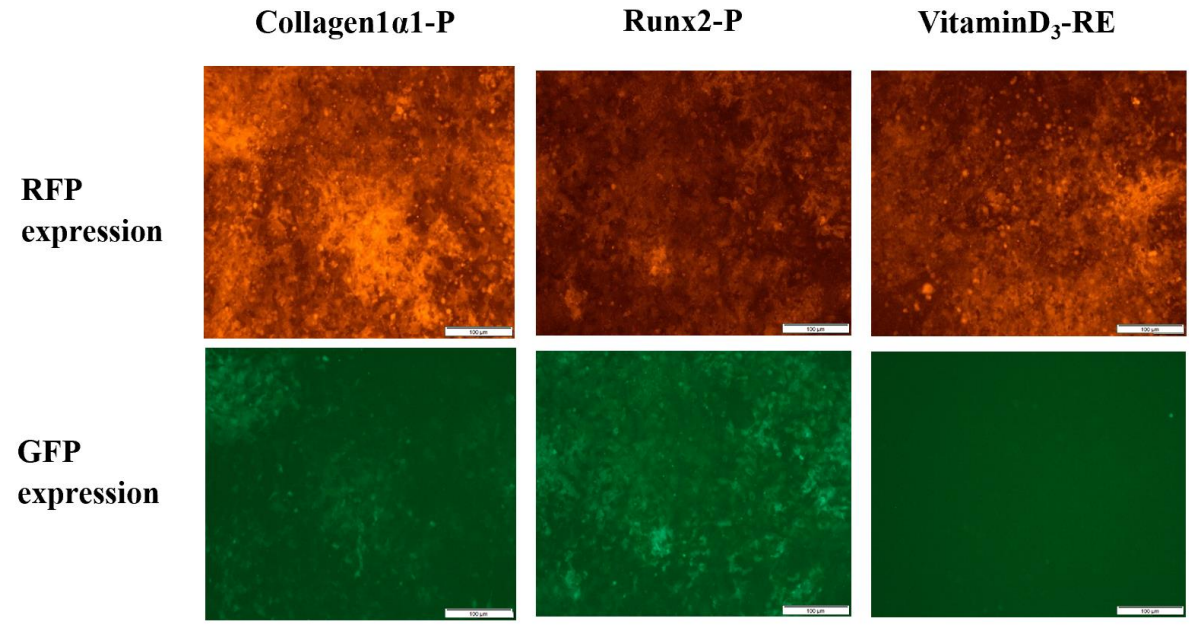


Figure 6. Saos2 cells immediately post-infection RFP+/GFP+. Infected Saos2 cells initially exhibited some degree of GFP expression under normal culture conditions. As such, RFP+/GFP- cells were FACs isolated, to then be placed under osteogenic conditions to visualise and quantify inducible GFP expression.

Saos2 cells infected with 3 LVDP reporters during induced osteogenesis

Capacity for inducible GFP expression under osteogenesis, was assessed over a 14-day time course. Initial studies used the human osteosarcoma cell line, Saos2 to determine the functional efficacy of the different fluorescence reporter constructs. Saos2 cells were infected with each reporter construct and fluorescence images were taken of each construct under control and osteogenic conditions showing minimal GFP expression for all constructs (Fig 7). This was

confirmed through flow analysis at the corresponding timepoints to quantify GFP induction over time.

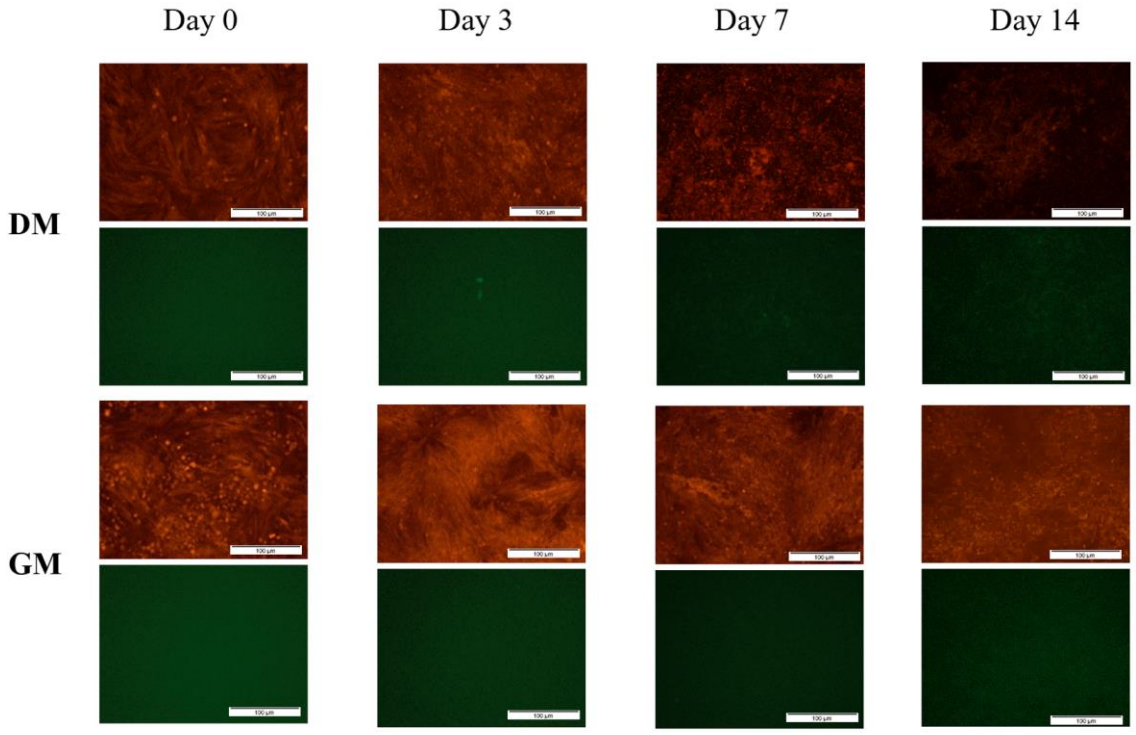
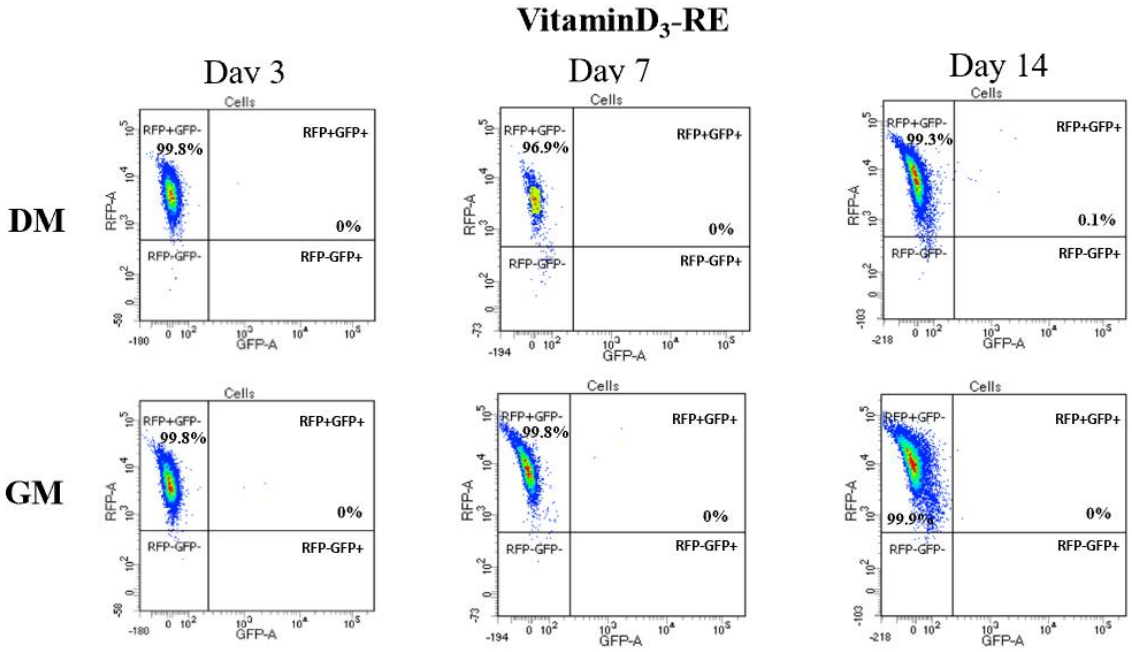


Figure 7. Saos2 cells infected with VitaminD3-RE in the presence of growth media (GM) and differentiation media (DM). Fluorescence images showed consistent RFP expression in both GM and DM groups, indicating high degree viral data normalization. Minimal GFP expression was observed in the control population as expected. Additionally, minimal GFP expression was also observed for cell population in the presence of DM.



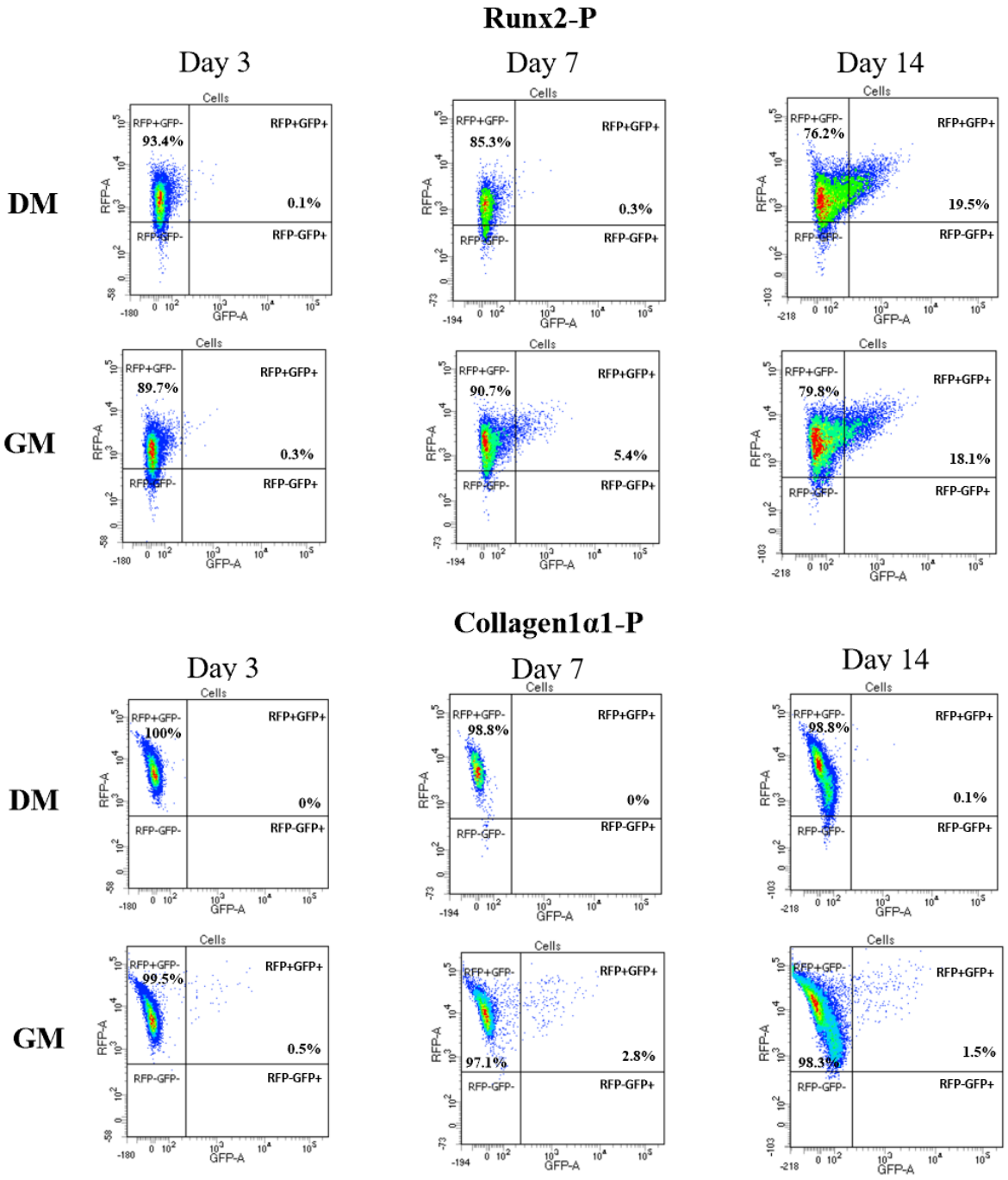


Figure 7.1. Corresponding flow analysis for observed time points during Saos2 osteogenic assay. Initial day 0 FACS sort yielded a population of RFP+/GFP- cells as represented in the upper left sorting gate in each graph, then seeded for osteogenesis induction. Quantified RFP/GFP expression showed no significant pathway activation under GM or DM at any time point.

Runx2-P LVDP construct infected immortalised human cranial suture progenitors (iSuP) shows some capacity for inducible GFP expression

An immortalised cranial suture cell line (iSuP) derived from a craniosynostosis patient was also infected with the different constructs (Fig 8). Post-infection, cells containing all tested constructs exhibited a degree of dual fluorescence under normal growth conditions and were thus isolated by FACS with the intention of obtaining a stable single RFP+/GFP- cell population. Collagen1α1-P infected cells indicated no significant GFP induction over 14-days under osteogenic inductive conditions (Fig 9). Runx2-P infected iSuPs showed approximately 26%, inducible GFP expression under osteogenic inductive condition by day 14, in contrast to vector control iSuPs which showed 4.4% cells GFP expression at the same time point. VitaminD_3-RE infected cells were highly RFP+/GFP+ under control conditions and seeded for osteogenesis, however no significant increase in GFP was present in DM relative to GM.

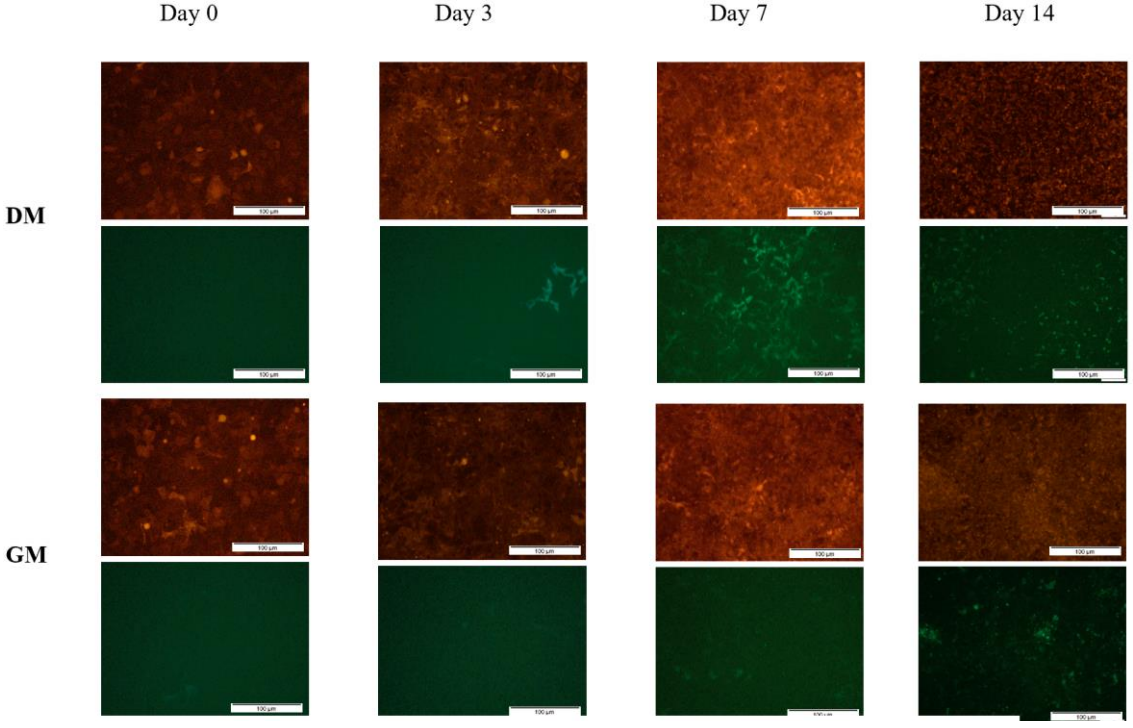
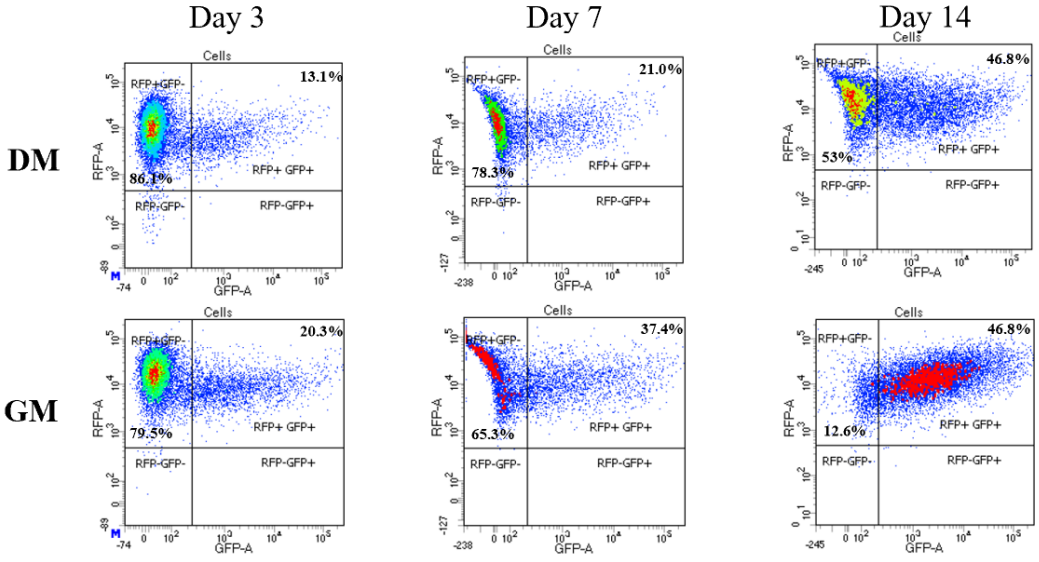
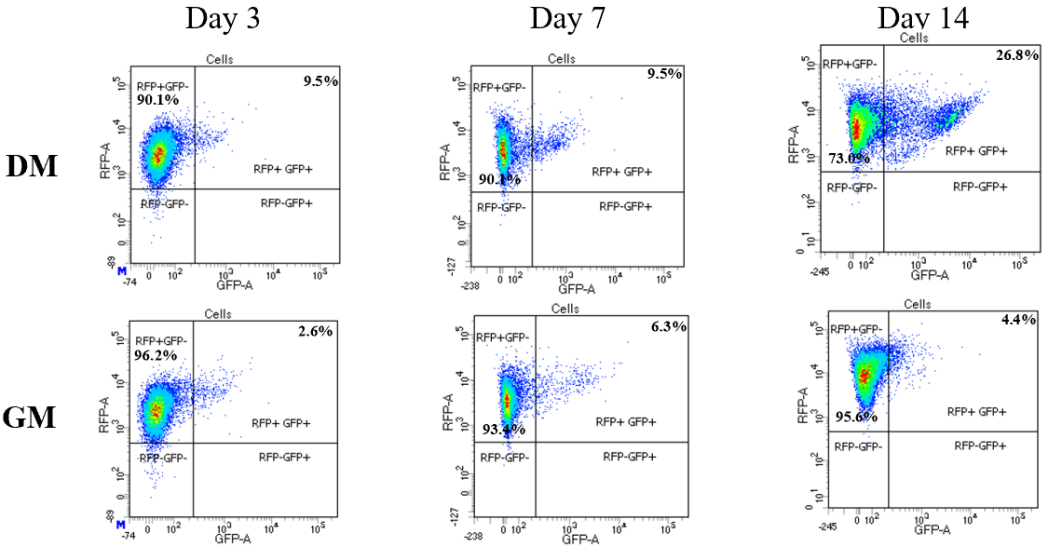


Figure 8. Fluorescence images of Runx2-P infected iSuPs under osteogenic conditions. Visually, an increase in GFP expressive cells is present after the day 3 time point. Conversely, minimal GFP expression in the control group relative to the osteogenic group was observed.

VitaminD$_3$-RE



Runx2-P



Collagen1α1-P

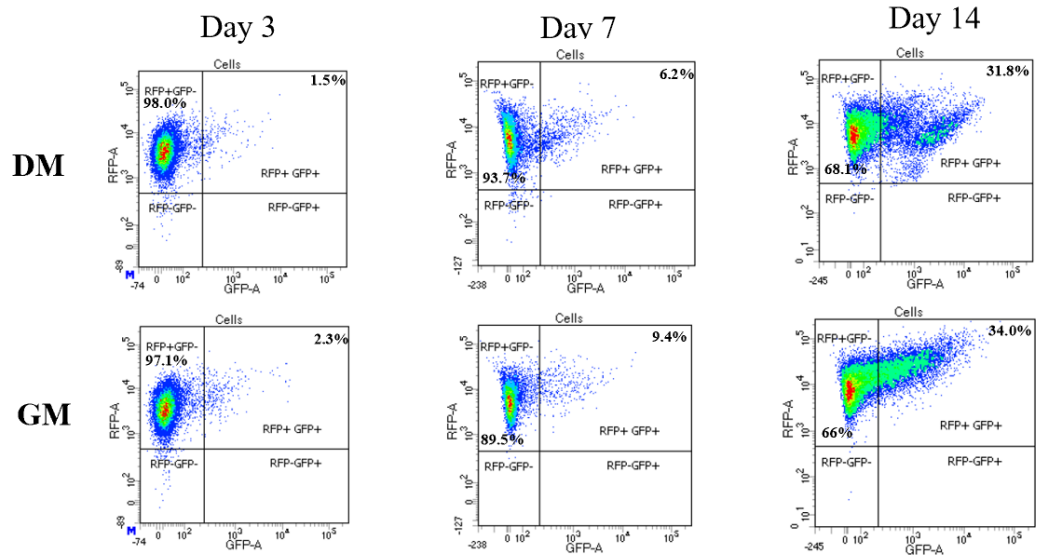


Figure 8.1 Flow analysis data of LVDP infected iSuPs under osteogenic conditions. An increase in GFP expressive cells is present after the day 7 time point in iSuPs infected with Runx2-P. Conversely, minimal GFP expression in the control group relative to the osteogenic group was observed. Data at day 14 indicated 26.8% of total single cells analysed were RFP+/GFP+ in DM, with corresponding GM treated cells consisting of 4.4% RFP+/GFP+ cells.

Gingival fibroblasts infected with VitaminD$_3$-RE display some capacity for GFP induction under osteogenesis

Human gingival fibroblasts were infected with the three different LVDP constructs and cultured under osteogenic conditions for 14 days. The gingival fibroblasts (n=3 donors) were isolated by FACS for RFP+/GFP- subpopulation prior to seeding under osteogenic inductive conditions. Donor GFD0003 proliferated slowly following the initial sort, and a stable population of single RFP+ cells was not able to be obtained before the cells became senescent. Other gingival fibroblast donors (n=2) were viable following FACS isolation. Despite treatment methods being uniform for the remaining gingival fibroblasts (n=2 donors), 1 donor was unable to be assessed via flow cytometry due to a lack of viable cells after osteogenesis was induced. Gingival fibroblasts (n=1 donors) infected with VitaminD$_3$-RE displayed an inducible ability to express GFP under osteogenic conditions (Fig 5.1), with no apparent inducible GFP expression observed in Collagen1a1-P and Runx2-P infected subpopulations.

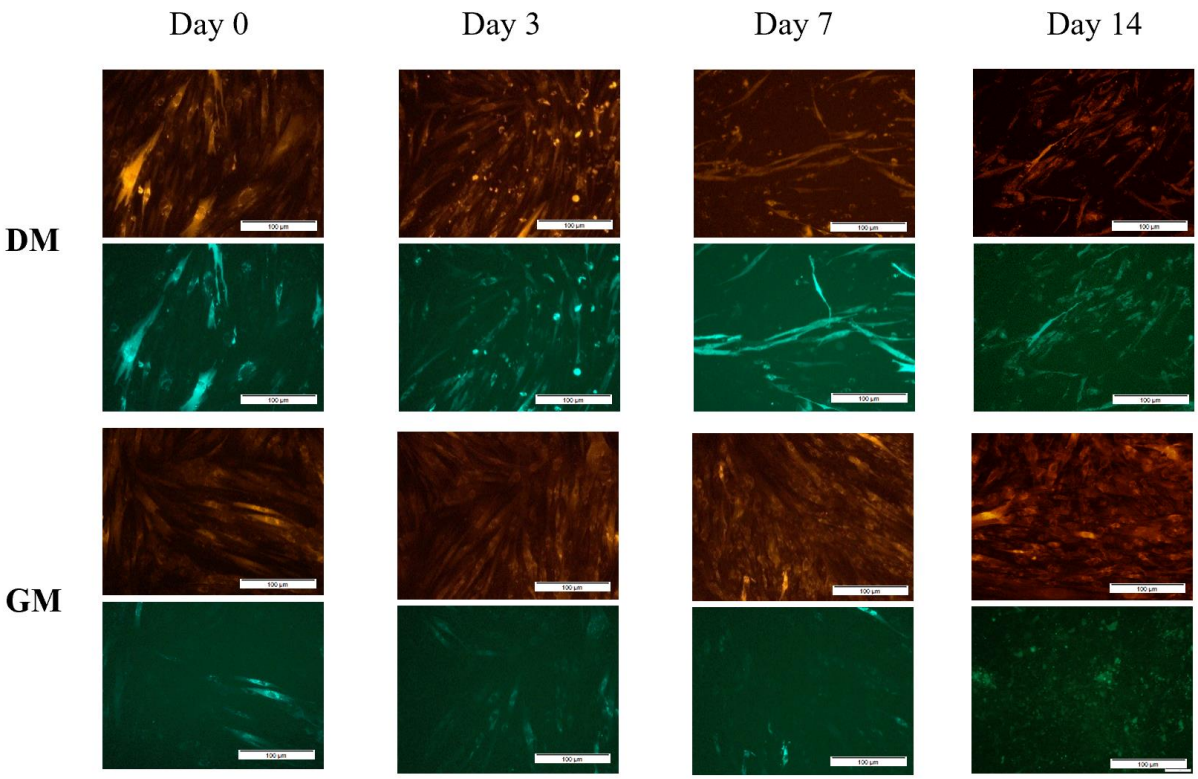
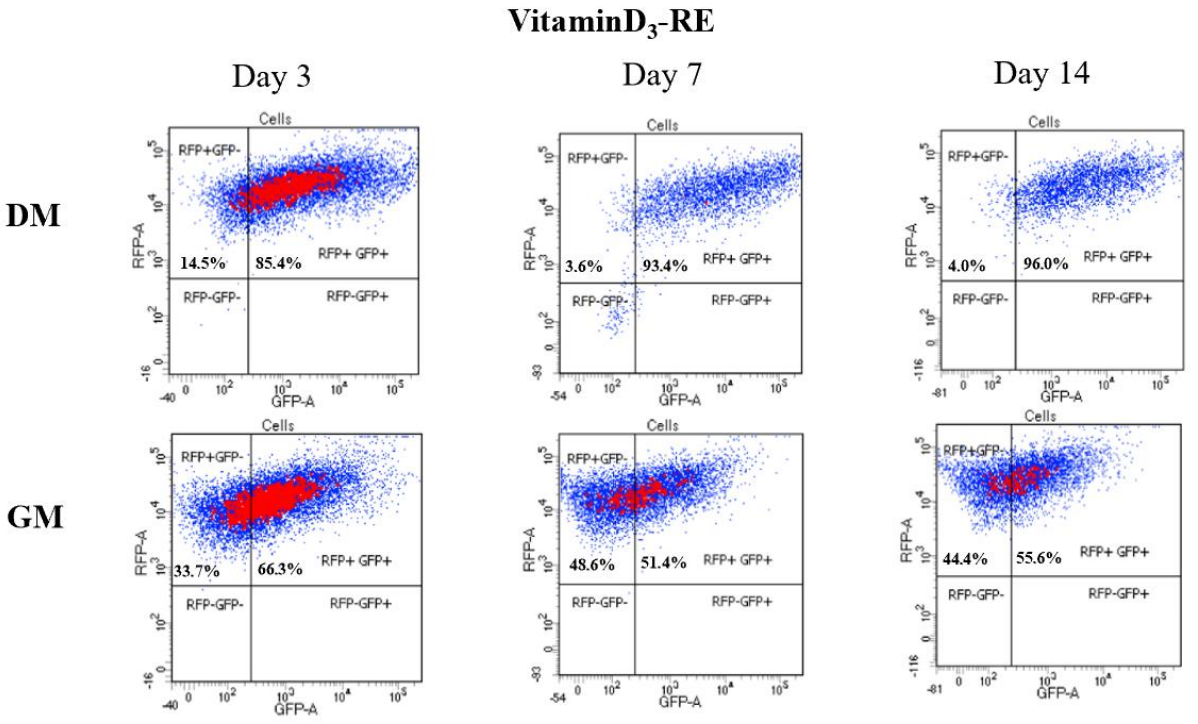


Figure 9. Fluorescence images of VitaminD$_3$-RE infected Gingival Fibroblasts (Donor HUGHF) under osteogenic conditions. VitaminD$_3$-RE infected cells appeared to have greater GFP expression under osteogenic conditions.



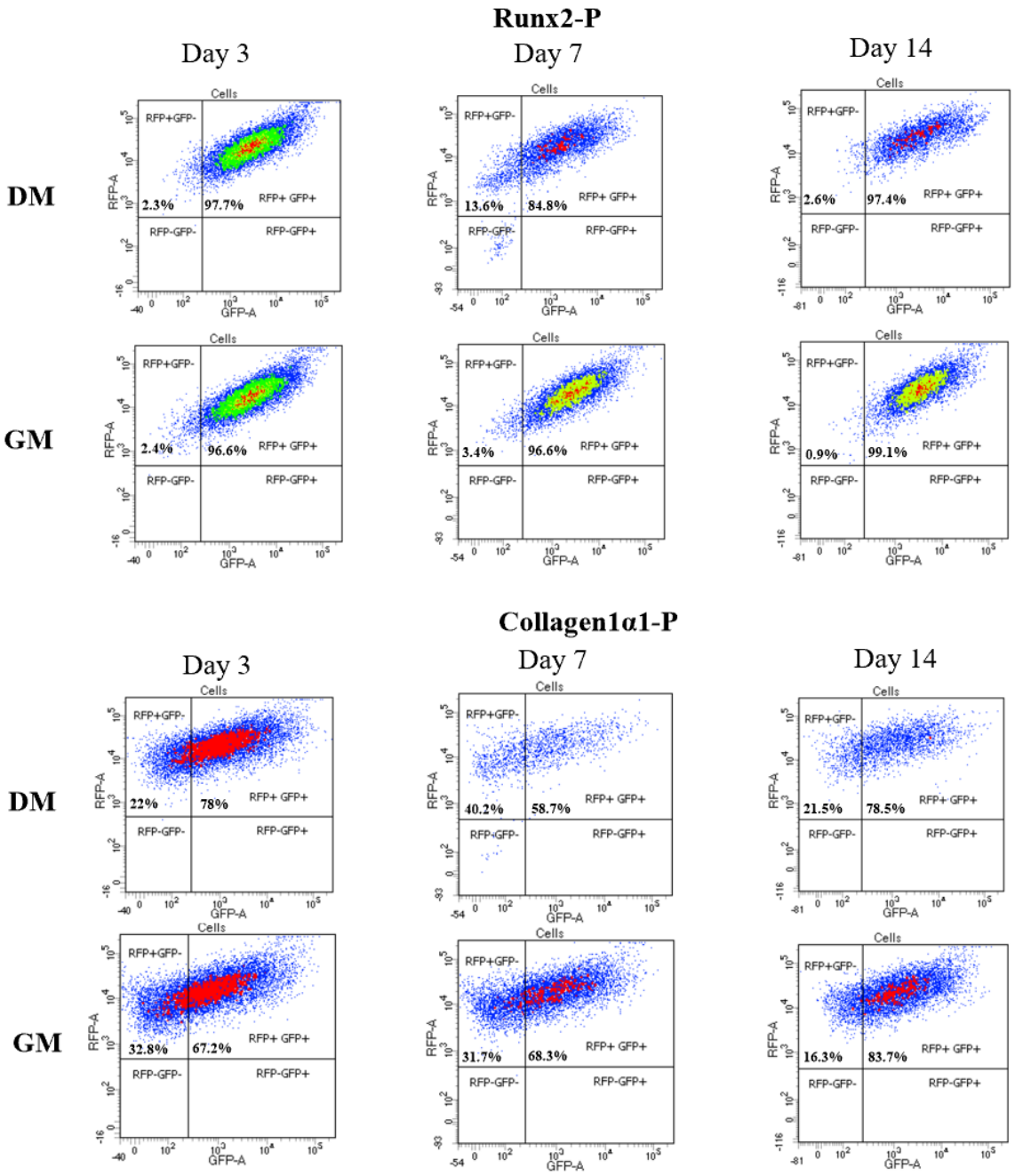


Figure 9.1. Flow graphs representing LVDP infected Gingival Fibroblasts (Donor HUGHF) under control and osteogenic conditions. As seen in *figure 5*, there is an apparent increase in dual fluorescence following the addition of DM to the cells in the VitaminD_3-RE group. At 14 days, flow data determined 96.0% of analysed cells to be RFP+/GFP+ under DM conditions, with 55.6% of cells cultured under normal GM conditions were dual-fluorescent.

Discussion

Saos2 cells and iSuPs are phenotypically osteoblastic[35,36,37], and gingival fibroblasts have limited capacity to form mineral under osteogenic conditions[38,39] This study has shown the potential inducibility of a Runx2-P LVDP fluorescent reporter in a craniosynostosis cell line under osteogenic conditions. Furthermore, the data demonstrated an increase in GFP expressing cells under osteogenic conditions implying that VitaminD_3-RE may be a suitable candidate in combination with gingival fibroblasts to screen a potential chemical library.

Had time permitted, an evaluation of endogenous transcription of Col1α1, Runx2 and VitaminD_3 would have been undertaken for each infected cell type. This data would indicate whether the infected cells expressed high levels of these genes under normal growth conditions or osteogenic inductive conditions. A more accurate conclusion of reporter activity could then be drawn. Cells tested that were highly GFP expressive under GM conditions were likely to have been committed to and osteoblastic lineage and therefore are expressive of these genes endogenously. This justified sorting cells based on observational data for RFP+/GFP- cells to then be induced down an osteogenic path. However, many tested groups remained GFP- under GM and DM conditions. This is likely attributed to the process of viral integration of reporter DNA into regions of dense heterochromatin in the GFP negative cells. Areas of dense heterochromatin are compacted, which drastically decreases transcription factor access to these regions, thus repressing transcription[40]. Future studies should involve single cell clonal selection of infected cells from the different cell lines that have a RFP+/GFP- phenotype under GM conditions and able to be osteogenically induced. A possible reason sorting based on RFP+/GFP- phenotype was ineffective is that the cells were already committed to an osteoblastic lineage as mentioned. Alternatively, viral constructs may have been integrated into regions of euchromatin which has loose chromatin and promotes transcription[41].

Primary human cranial suture cells have been immortalised via the introduction of an SV40 T antigen, thus creating an immortalised cranial progenitor line which will not senesce upon reaching high passage numbers compared to primary cells[37]. This was highly relevant to the study, as these cells were derived from the unfused cranial sutures of craniosynostosis patients. An increase in GFP expressive in DM relative to GM was observed in iSuPs infected with Runx2-P. This is potentially due to less dense heterochromatin present in iSuPs compared to Saos2 cells, hence having a greater repressive effect on the transcription of the tested transcription factors. Due to the highly osteogenic potential of iSuPs compared to Saos2 cells, they may express greater levels of Runx2[37]. Alizarin red staining and calcium assay to assess levels of cellular mineral at each time point should also be conducted as further confirmation of osteogenic activity.

In craniosynostosis, studies have shown that osteoinductive grown factor Nell-1 is tightly regulated by master transcription factor, Runx2. Such studies have also shown Nell-1 to be present throughout the fusion process of the cranium[42]. Additionally, overexpression of Nell-1 is associated with significant cranium malformations in a murine model[42]. This may explain results why Runx2-P induced GFP expression following the induction of osteogenesis in iSuPs was found to increase relative to normal growth conditions. Therefore, selection of clonal populations of infected cells may alleviate this issue. Saos2 is an osteosarcoma cell line, with an osteoblast progenitor-like phenotype. Due to the nature of such cell lines, Saos2 cells are likely to contain a chromosome duplications, deletions/rearrangements and a multitude of mutations acquired over time due to the high number of cell passages. In turn this may alter the expression of genes which activate the reporter constructs. The chromosome number in Saso2 cells is hypotriploid, with each cell containing an average 56 chromosomes[35,36]. Additionally, a vast majority of chromosome complement contains complex rearranged chromosomes. This might explain why this cell line had high osteogenic reporter expression in GM with no

significant induction in DM. Changes in heterochromatin and euchromatin after the cells have been infected may also alter the expression of constructs if the reporters are integrated into areas of transcriptional repression[42]. Furthermore, fibroblasts are among the most prevalent stromal cells existing within human connective tissue[43]. Gingival fibroblasts are not phenotypically osteoblastic. Based on this, they were selected as a negative control which may reduce double positivity upon initial infection. Research has shown that a subset of gingival fibroblasts have a limited osteogenic capacity[38,39]. This is in contrast to bone marrow derived stromal cells that differentiate into pre-osteoblasts following cell culture which may affect the expression of the osteogenic reporters in the viral constructs[44].

Immortalised human cranial suture progenitor cells appear to have some potential for inducible expression of Runx2-P. Based on initial observational data, future research will include a greater number of replicates. Isolating clonal Runx2-P infected iSuP cells to further identify a subpopulation with the lowest GFP expression in GM and the highest GFP expression under DM may be an appropriate candidate to use to screen a chemical library of inhibitors. This combination of cell type and construct may be the first step in developing an effective drug-based treatment for craniosynostosis, something not as of yet achieved through any known regulators of osteogenesis.

The main limitation of this study has been largely the result of the Covid-19 global pandemic. Due to social distancing measures taken to slow the national spread of the pandemic, a significant period of potential research time was lost. As such, time permitted only the partial completion of aim 1 as previously outlined. Adequate research time would have allowed a greater volume of lab work to be undertaken, thus increasing result output and overall data generation. Moreover, various functional assays to assess cell mineralisation under osteogenic conditions were unable to be effectively undertaken. Future research should therefore include more replicates of experiments conducted for statistical confirmation of result significance.

Another limitation of this study was the consistently double positivity of infected cells under control conditions. The design of the LVDP constructs is such that insulator sequences between the two promoter regions minimise interference. Cells assessed for screen suitability in this study all share commitment to an osteoblastic lineage. Therefore, it is likely that dual-fluorescence observed is the result of existing endogenous osteogenic gene activation in a normal growth setting which remains to be assessed. Furthermore, cell lines used (Saos2 and iSuP) may possess potential mutations promoting an osteoblastic phenotype hence promoting expression of the P/RE of the construct. In future research, isolating a single RFP+/GFP- stable clonal population may be a potential avenue due to the immortality of these cells, which is not possible in primary cells due to their limited life-span in vitro.

Conclusions

The work done in this study is a fundamental first step in identifying a novel epigenetic regulator of osteogenesis and its inhibitor in craniosynostosis. This is the first study in which LVDP reporter constructs have been used to infect a variety of cell types in order to identify a suitable human craniosynostosis bone model through which a chemical screen may be performed. Within this study we have identified that iSuPs infected with Runx2-P show some capacity for inducible GFP expression when undergoing osteogenesis. Moreover, gingival fibroblasts have also demonstrated some GFP induction when infected with a VitaminD_3-RE. However, more replicates of these experiments must be performed in conjunction with function assays and endogenous gene expression assessment to improve statistical power. Additionally, it has also been shown that single sorting of infected cells is insufficient when attempting to isolate a stable population of RFP+/GFP- cells. Future experiments will involve single cell clonal selection of Runx2 reporter infected iSuP cells to identify clones with a low GFP in GM conditions and highly inducible GFP in DM conditions. As such, the results of this experiment has furthered the goal identifying a suitable cell type/reporter combination that may be used

for a chemical screen of potential inhibitors. Using this research as a groundwork, a novel epigenetic regulator of osteogenesis in craniosynostosis may be identified thereby uncovering a potential drug-based therapeutic target for the treatment of craniosynostosis.

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References:

1. Zhao H, Feng J, Ho TV et al. The suture provides a niche for MSC. Nat Cell Biol. 2015;17:386.

2. Schurmann, M., Brotzmann, V., Butow, et al. 2018. Identification of a Novel High Yielding Source of Multipotent Adult Human Neural Crest-Derived Stem Cells. *Stem Cell Reviews and Reports,* 14**,** 277-285.

3. Johnson D, Iseki S, Wilkie AO, et al. Expression patterns of Twist and Fgfr1, -2 and -3 in the developing mouse coronal suture.. 2000;91:341.

4. Miraouichi, H. & Marie, P. J. 2010. Pivotal role of Twist in skeletal biology and pathology. *Gene,* 468**,** 1-7.

5. Cohen MMJ. Diagnosis, Evaluation, and Management In: Cohen MMJM, R.E., ed. Craniosynostosis. New York: Oxford University Press; 2000:11.

6. Behr B, Longaker MT, Quarto N. Craniosynostosis of coronal suture in twist1 mice occurs through endochondral ossification. Front Physiol. 2011;2:37.

7. Coussens AK, Wilkinson CR, Hughes IP, et al. Unravelling the molecular control of calvarial suture fusion in children with craniosynostosis. BMC Genomics. 2007;8:458.

8. Chen ZF, Behringer, RR. twist is required in head mesenchyme.. Genes Dev. 1995;9:689.

9. Howard TD, Paznekas WA, Green ED, et al. Mutations in TWIST, a bHLH transcription factor, in Saethre-Chotzen syndrome. Nature genetics. 1997;15:36.

10. Murre C MPS, Vaessin H, Caudy M et al. Interactions between heterologous HLH proteins generate complexes that bind specifically to a common DNA sequence. Cell. 1989;58:537.

11. Menicanin D, Bartold PM, Zannettino AC, et al. Identification of a common gene expression signature associated with immature clonal mesenchymal cell.. Stem Cells Dev. 2010;19:1501.

12. Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A. & Simmons, P. J. 2003. Molecular and cellular characterisation of highly purified stromal cells derived from human bone marrow. *J Cell Sci,* 116, 1827-35

13. G, M. C., Wu, N. L., Roybal, P. G., Sun, J., Liu, L., Yen, Y. & Maxson, R. E., Jr. 2009. EphA4 as an effector of Twist1 in the guidance of osteogenic precursor cells during calvarial bone growth and in craniosynostosis. *Development,* 136**,** 855-64.

14. Cakouros D, Isenmann S, Cooper L, et al. Twist-1 induces Ezh2 recruitment regulating histonemethylation along the Ink4A/Arf locus in MSC. Molecular Cellular Biol. 2012;32:8.

15. Camp, E., Anderson, P. J., Zannettino, A. C. W., et al. 2018. Tyrosine kinase receptor c-ros-oncogene 1 inhibition alleviates aberrant bone formation of TWIST-1 haploinsufficient calvarial cells from Saethre-Chotzen syndrome patients. *J Cell Physiol,* 233, 7320-7332

16. Yousfi M, Lasmoles F, El Ghouzzi V, et al. Twist haploinsufficiency in Saethre-Chotzen syndrome induces calvarial osteoblast apoptosis.. Hum Mol Genet. 2002;11:359.13**.**

17. Im GI & Shin KJ (2015). Epigenetic approaches to regeneration of bone and cartilage from stem cells. Expert Opin Biol Ther 15, 181-193

18. Yi, S.-J., Lee, H., Lee, J., Lee, K., Kim, J., Kim, Y., Park, J.-I. & Kim, K. 2019. Bone Remodeling: Histone Modifications as Fate Determinants of Bone Cell Differentiation. *International Journal of Molecular Sciences,* 20**,** 3147.

19. Bannister, A. J., Kouzarides, T. 2001. Regulation of chromatin by histone modifications. *Cell Research,* 21, 381-395.

20. Ren, J., Huang, D., Li, R., et al. 2020. Control of mesenchymal stem cell biology by histone modifications. *Cell & Bioscience,* 10, 11.

21. Hemming S, Cakouros D, Codrington J, et al. EZH2 deletion in early mesenchyme compromises postnatal bone microarchitecture..FASEB J. 2016. Dec 1. pii: fj.201600748R.

22. Ozkul Y & Galderisi U (2016). The impact of epigenetics on mesenchymal stem cell biology. J Cell Physiol 231, 2393-2401.

23. Dudakovic A, Camilleri ET, Riester SM, et al. EZH2 Inhibition Stimulates Bone Formation and Mitigates Bone Loss Caused by Ovariectomy in Mice. J Biol Chem. 2016;291:24594.

24. Hemming, S., Cakouros, D., Isenmann, S, et al. 2014. EZH2 and KDM6A act as an Epigenetic Switch to Regulate Mesenchymal Stem Cell Lineage Specification. Stem cells (Dayton, Ohio), 32.

25. Rojas, A., Aguilar, R., Henriquez, B., Lian, J. B., Stein, J. L., Stein, G. S., Van Wijnen, A. J., Van Zundert, B., Allende, M. L. & Montecino, M. 2015. Epigenetic Control of the Bone master Runx2 Gene during Osteoblast-lineage Commitment by the Histone Demethylase JARID1B/KDM5B. *J BiolChem,* 290**,** 28329-42.

26. Kim, W. J., Shin, H. L., Kim, B. S., Kim, H. J. & Ryoo, H. M. 2020. RUNX2-modifying enzymes: therapeutic targets for bone diseases. *Experimental and Molecular Medicine,* 52**,** 1178-1184.

27. Somaiah, C., Kumar, A., Mawrie, D., Sharma, A., Patil, S. D., Bhattacharyya, J.,Swaminathan, R. & Jaganathan, B. G. 2015. Collagen Promotes Higher Adhesion, Survival and Proliferation of Mesenchymal Stem Cells. *PLoS One,* 10**,** e0145068.

28. Vater, C., Kasten, P. & Stiehler, M. 2011. Culture media for the differentiation of Mesenchymal stromal cells. *Acta Biomater,* 7**,** 463-77.

29. Hanna, H., Mir, L. M. & Andre, F. M. 2018. In vitro osteoblastic differentiation of mesenchymal stem cells generates cell layers with distinct properties. *Stem Cell Research & Therapy,* 9**,** 203.

30. Van De Peppel, J. & Van Leeuwen, J. P. T. M. 2014. Vitamin D and gene networks in human osteoblasts. *Frontiers in Physiology,* 5.

31. Lou, Y.-R., Toh, T. C., Tee, Y. H. & Yu, H. 2017. 25-Hydroxyvitamin D(3) induces osteogenic differentiation of human mesenchymal stem cells. *Scientific reports,* 7**,** 42816-42816.

32. Wang, D., Song, J. & Ma, H. 2018. An in vitro Experimental Insight into the Osteoblast Responses to Vitamin D3 and Its Metabolites. *Pharmacology,* 101**,** 225-235.

33. Woeckel, V. J., Van der Eerden, B. C. J., Schreuders-Koedam, M., Eijken, M. & Van Leeuwen, J. P. T.M. 2013. 1α,25-dihydroxyvitamin D3 stimulates activin A production to fine-tune osteoblast-induced mineralization. *Journal of Cellular Physiology,* 228**,** 2167-2174.

34. Padmashali, R. M., Mistriotis, P., Liang. 2014. Lentiviral Arrays for Live-cell Dynamic Monitoring of Gene and Pathway Activity During Stem Cell Differentiation. Molecular Therapy, 22, 1971-1982.

35. Rodan, S. B., Imai, Y., Thiede, M. A., Wesolowski, G., Thompson, D., Bar-Shavit, Z., Shull, S., Mann, K. & Rodan, G. A. 1987. Charaterization of a human osteosarcoma cell line (SAOS-2) with osteoblastic properties. *Cancer research,* 47**,** 4961-6.

36. Fogh J. Human tumor cells in vitro. New York: Plenum Press; 1975, *p136.*

37. Song, D., Zhang, F., Reid, R. et al. 2017. BMP9 induces osteogenesis and adipogenesis in the immortalized human cranial suture progenitors from the patent sutures of craniosynostosis patients. *Journal of Cellular and Molecular Medicine,* 21**,** 2782-2795.

38. Nebel, D., Svensson, D., Arosenius, K., Larsson, E., Jönsson, D. & Nilsson, B.-O. 2015. 1α,25-dihydroxyvitamin D3 promotes osteogenic activity and downregulates proinflammatory cytokine expression in human periodontal ligament cells. *Journal of Periodontal Research,* 50**,** 666-673.

39. Posa, F., Di Benedetto, A., Colaianni, G., Cavalcanti-Adam, E. A., Brunetti, G., Porro, C., Trotta, T., Grano, M. & Mori, G. 2016. Vitamin D Effects on Osteoblastic Differentiation of Mesenchymal Stem Cells from Dental Tissues. *Stem Cells International,* 2016**,** 9150819.

40. Gordon, J.A.R.; Stein, J.L., Westendorf, J.J., van Wijnen, A.J. 2015. Chromatin modifiers and histone modifications in bone formation, regeneration, and therapeutic intervention for bone-related disease. *Bone, 81,* 739–745

41. Zhou, P., Wu, E., Alam, H.B., Li, Y. 2014.Histone cleavage as a mechanism for epigenetic regulation: Current insights and perspectives. *Curr. Mol. Med., 14,* 1164–1172

42. Zhang, X., Zara, J., Siu, R. K., Ting, K. & Soo, C. 2010. The Tole of NELL-1, a Growth Factor Associated with Craniosynostosis, in Promoting Bone Regeneration. *Journal of dental research,* 89,

865-78.

43. Zhang, Q. Z., Ngyuen, A. L., YU, W. H. & Le, A. D. 2012. Human oral mucosa and gingiva: a unique reservoir for mesenchymal stem cells. *Journal of dental research,* 91**,** 1011-1018.

44. WeiY, Y-HChen, L-YLi, J Lang, S-P Yeh, B Shi, C-C Yang, J-Y Yang, C-Y Lin, C-C Lai and M-C Hung. (2010). CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. Nat Cell Biol 13:87–94.