**Aus der Poliklinik für Zahnerhaltung und Parodontologie**

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# The effects of low molecular weight hyaluronan on the cementogenic, ligamentogenic and osteogenic differentiation of human mesenchymal stem cells (hMSCs) and periodontal ligament cells (PDL-hTERTs)

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To Mike Guang and My Parents

for their love and support

# TABLE OF CONTENTS

# ABSTRACT

Periodontitis is a highly prevalent chronic inflammatory oral disease. Hyaluronan (HA) is a non-sulfated glycosaminoglycan which helps anti-inflammatory and tissue repair. The aim of this study was to delineate the effects of various sized HA molecules on periodontal cells. Oligosaccharide nano HA and 150 kDA HA were used to stimulate human mesenchymal stem cells (hMSCs) and human periodontal ligament cells with human telomerase reverse transcriptase (PDLhTERTs), for 3, 7 and 21 days. HA receptors, CD44, receptor of hyaluronic acid mediated motility (CD168) and the Toll-like receptor (TLR)-4, have been substantially expressed in both cell types. In hMSCs CD44 and CD168 expression remained roughly unchanged during the entire observation period; in PDLhTERTs nano and/or the 150 kDa HA fragment significantly attenuated the expression of the CD44 and CD168 receptors. TLR4 expression was inhibited by nano and/or 150kDa HA in both cell types at day 21. The presence of HA reduced the transcription of the cementogenic markers, cementum-derived attachment protein (CAP) and cementum protein 1 (CEMP1), in both cell types, especially nano HA. Scleraxis (SCX), a ligamentogenic marker, remained almost unchanged irrespective of the specific stimulation condition. Early stage osteogenic marker alkaline phosphatase (ALP) was induced by the various stimulation conditions in both hMSCs and PDLhTERTs and stronger in the presence of nano and 150 kDa HA. Bone sialoprotein (BSP) remained roughly unchanged under stimulation. Osteogenic markers collagen type I alpha 1 (COL1A1) in both cell types and osteocalcin (OCN) in hMSCs were also enhanced by the HA fragments.

However in PDLhTERTs OCN was inhibited by 150k HA. The osteogenic stimulation alone and together with HA lead to the highest calcium deposition.

Taken together the current study revealed that small HA fragments cause differential effects on hMSCs and PDLhTERTs. Nano HA seemed to have more positive effects in osteogenic differentiation than 150kDa HA. These fragments seem to enhance the earlier steps of osteogenic differentiation in both types of stem cells but impair the expression of cementogenic differentiation markers and the mineralization of the ECM during osteogenesis within 21 days. Since the expression of scleraxis was unaffected HA seems to have no influence on the ligamentogenesis.

# 1. Introduction

Periodontitis is a highly prevalent chronic inflammatory oral disease which not only leads to bone loss, attachment loss, and tooth loss but also has negative effects for the whole body (Hajishengallis 2015). To cure periodontitis, achieve periodontal regeneration, needs to reconstruct alveolar bone, connective tissue, cementum and periodontal ligament (PDL). Hyaluronan (HA) has influence on tissue repair and has already been widely used in medical applications (Tolg et al. 2014, Robert 2015). This study aimed to delineate the effects of various sized HA molecules on human periodontal ligament cells (PDL-hTERT) and mesenchymal stem cells (hMSCs) regeneration.

## 1.1 HA

HA, also called hyaluronic acid, is a non-sulfated glycosaminoglycan. Karl Meyer isolated it from vitreous humor first time in 1934 (Meyer et al. 1934). HA can be found in all tissue of vertebrates, especially in extracellular matrix (ECM) of skin and connective tissues (Laurent et al. 1992, Fraser et al. 1997).

## 1.2 HMW-HA and LMW-HA

The molecular weight of HA shows considerable variability. Native HA exists as high-molecular weight HA (HMW-HA) and its typical molecular weight is $>10^6$ Da (Noble 2002). In addition, low molecular weight HA (LMW-HA) fragments are generated as a result of enzymatic activity during HA synthesis or degradation

mediated by hyaluronidases or chemical activity triggered by reactive oxygen species (Jiang et al. 2007, Kavasi et al. 2017). Enzymatic fragmentation of HMW-HA is particularly controlled by hyaluronidase-1 and -2 (Hyal-1 and -2). The membrane bound Hyal-2 splices hyaluronan to fragments of 20 kDa. Following endocytosis these fragments are subjected to further lysosomal digestion by Hyal-1 (Litwiniuk et al. 2016). Commonly the LMW-HA fragments show a highly disperse molecular size with overlapping lengths ranging from small oligosaccharides (4mer) to < 500.000 Da (Petrey et al. 2014). Depending on the molecular weight HA has different and partially antagonistic biological effects (Stern et al. 2006). High molecular weight HA promotes tissue homeostasis and inhibits angiogenesis, shows anti-inflammatory and immunosuppressive properties and inhibits the proliferation of many cell types (Gao et al. 2010, Ghosh et al. 2015). On the contrary low molecular weight HA has been suggested to act as danger signal within damaged tissues (Powell et al. 2005). Once the tissue homeostasis is disrupted, e.g. upon inflammation, tissue injury and tumor invasion the native HMW-HA is degraded into smaller fragments. Both, the loss of native HMW-HA and the increasing amount of low molecular weight HA can induce changes in cell behavior and signaling (Yang et al. 2012). It has been shown in several experimental models that LMW-HA has mitogenic effects and enhances cell proliferation (David-Raoudi et al. 2008). Compared to HMW-HA the smaller HA fragments exert potent pro-inflammatory and immunostimulatory effects (Wang et al. 2011, Litwiniuk et al. 2016). Specifically, the very small oligosaccharides seem to amplify the signals induced by the presence of small to intermediate-sized fragments

of HA (Aya et al. 2014).

Due to the effects on cell proliferation and its almost ubiquitous occurrence, HA at different molecular weights has been proposed to play a significant role in healing of damaged tissue (Jiang et al. 2007, Tolg et al. 2014). Considering the cellular and molecular mechanisms involved in wound healing HA induces different effects depending on its molecular size (Kavasi et al. 2017). The concentration of HMW-HA sharply increases during the earliest phase of wound healing which is degraded afterwards leading to the accumulation of LMW-HA. Apart from the induction and enhancement of inflammatory reactions functional significance of the HA turnover might comprise the stimulation of keratinocytes and fibroblasts during wound healing (Tolg et al. 2014, D'Agostino et al. 2015). In intervertebral disc cells specifically HA oligosaccharides up regulated various matrix repair genes, i.e. ACAN, COL1A1 and COL2A1 (Fuller et al. 2016). Inline with that, the presence of 6mer and 8mer oligosaccharides induces a considerably stronger migration of rat dermal fibroblasts resulting in a more rapid closure of experimental excision wounds as compared to larger HA fragments of 40 kDa (Tolg et al. 2014).

## 1.3 HA receptors

HA has many receptors, including CD44, CD168, Toll-like receptor 4 (TLR4) , Toll-like receptor-2 (TLR2) , intercellular adhesion molecule-1 (ICAM-1), Lymphatic Vessel Endothelial hyaluronan receptor 1 (LYVE 1) and so on (Vigetti et al. 2014). CD44 and CD168 are related with cell proliferation, migration and tumorigenesis.

TLR2 and TLR 4 are related with inflammation, cell survival and apoptosis. CD44, CD168 and TLR4 are relative to wound healing, infection and tissue recognition, therefore they were chosen to be tested in this research.

### 1.3.1 CD44

CD44 is the main receptor for the perception and mediation of the HA signal (Wang et al. 2011, Yang et al. 2012). As a result of alternative splicing and post-translational modifications it occurs in various isoforms in the membrane of many human cells showing different affinity for HA (Aruffo et al. 1990, Tammi et al. 1998). CD44 is not only important for the interactions between cells and the ECM but also for the intercellular interactions (Bajorath 2000). It was shown that CD44 is highly expressed in the dermal and epithelial compartment of the human skin (Wang et al. 1992). In keratinocytes the CD44 receptor seems to mediate various central functions in maintaining tissue homeostasis and repair, i.e. binding to growth factors and intercellular adhesion (Bourguignon 2014, Kavasi et al. 2017). Moreover, the CD44 receptor seems to be involved in the HA induced differentiation of monocytes into fibrocytes during wound repair (Maharjan et al. 2011).

### 1.3.2 CD168

CD168, also named RHAMM (receptor for hyaluronan-mediated motility), is the second major HA receptor that is present in numerous cell types (Entwistle et al. 1996, Croce et al. 2003). CD168 contributes to fibroblast migration, differentiation

and wound repair (Tolg et al. 2006). HA requires CD168 in modulating growth factor induced mammary gland branching (Tolg et al. 2017). Compared with benign tissues, malignant tumors have higher HA deposition. HA predicts tumor progression in some tumor types and affects tumorigenesis and tumor aggressiveness (Nikitovic et al. 2013, Vigetti et al. 2014). CD168 regulates LMW-HA via a beta-catenin/c-myc signaling axis and, for example, suppresses fibrosarcoma cell proliferation (Kouvidi et al. 2016).

### 1.3.3 TLR-4

In the ECM of injured or inflamed tissue LMW-HA will be broken down into low molecular weight fragments, which can stimulate epithelial cells and promote injury recognition through TLR4 (Taylor et al. 2004, Jiang et al. 2005). Though CD44 is the main receptor of HA, it is not required for HA to stimulate pro-inflammatory chemokines through TLRs. A study of lung injury showed that, in CD44-null mice LMW-HA can induce skin self-defense to protect cutaneous tissue from infection through release of β-defensin 2 by mediation of TLR2 and TLR4 (Gariboldi et al. 2008). Compared with TLR2, TLR4 can recognize lipopolysaccharide (LPS) pattern better (Takeuchi et al. 1999, Park et al. 2013, Mukherjee et al. 2016). LPS pattern is one of the progenitors to periodontitis (Dumitrescu et al. 2004), the most popular oral disease which leads to periodontal damage. This study aims to figure out HA's effect on inflamed periodontal regeneration, therefore TLR4 was chosen in this research.

## 1.4 Cells, markers and periodontal regeneration

Periodontal cells human mesenchymal stem cell (hMSC) and human periodontal ligament cells with human telomerase reverse transcriptase (PDLhTERTs) were chosen in this research.

### 1.4.1 hMSC

hMSCs exist extensively in all human tissues. They are mostly found in adult bone marrow (Caplan 1991, Bianco 2014), and, among others, can also be found in tooth pulp and periodontal tissues (Gronthos et al. 2000, Egusa et al. 2012). They are multipotential cells and can regenerate to several kinds of tissues such as bone, tendon, cartilage, ligament, muscle, endothelium, and epithelial cells (Pittenger et al. 1999, Jiang et al. 2002, Reyes et al. 2002). hMSCs can also produce growth factors and various cytokines, for instance bone morphogenetic protein (BMP) and transforming growth factor β 1 (TGF-β1), which can induce bone, cartilage, and tendon repair (Nixon et al. 2007, Borakati et al. 2018). Moreover, they are low immunogenicity cells that can be tolerated by the immune system and will home and migrate to damaged tissues when injured or inflamed (Rasmusson et al. 2007, Rasmusson et al. 2007, Rustad et al. 2012). Conclusively, MSCs are able to regenerate periodontal tissues and MSCs stem-cell-based therapy are widely used in several clinical disciplines (Egusa et al. 2012, Monsarrat et al. 2014).

### 1.4.2 PDLhTERT

PDLhTERTs are a periodontal ligament cell (PDL) derived immortalized cell line. They were transferred with lentivirus human telomerase reverse transcriptase (hTERT) and share the same characteristic as primary PDL cells (Docheva et al. 2010, Zhu et al. 2015). PDLhTERTs are hMSC-like cells since they can express some hMSC markers. They can also differentiate into osteoblasts, cementoblasts, adipocytes, and chondrocytes. Moreover, they can express osteoblast-related genes such as alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen, osteocalcin (OCN) and they can also promote periodontal regeneration (Mizuno et al. 2001, Seo et al. 2004, Huang et al. 2009, Wada et al. 2009, Docheva et al. 2010). PDLhTERTs can also express tendon related gene scleraxis (SCX) (Docheva et al. 2010).

### 1.4.3 Periodontal regeneration and HA receptors

Periodontitis as a highly prevalent oral disease mostly found in adults leads to bone loss, attachment loss and ultimately to tooth loss. To achieve periodontal regeneration needs to reconstruct alveolar bone, connective tissue, cementum and periodontal ligament. Stem cells such as hMSCs and MSC-like PDL cells, which are rich in periodontal tissues, are reported to be able to rebuild bone, cementum, collagen, ligament and, thus, to contribute to periodontal regeneration (Seo et al. 2004, Egusa et al. 2012, Tomokiyo et al. 2012).

hMSCs and PDL cells express both CD44 and CD168 receptors (Entwistle et al. 1996, Bian et al. 2013). It would be interesting to test the different cells, hMSCs and

PDL cells, under the stimulation of different molecular weight HA. And these would be helpful in making HA treatment a promising strategy for tissue regeneration (Ishikawa et al. 2014, Litwiniuk et al. 2016).

The proliferation and mineralization of human PDL cells has been shown to be linked to the expression of the CD44 receptor (Yeh et al. 2014). Several studies have found elevated levels of HA fragments in the gingival crevicular fluid at periodontally affected sites depending on the inflammatory activity (Utoh et al. 1998, Yan et al. 2000). Since the gingival crevicular fluid comprises a transudate or exudate of the blood serum and due to the inflammatory condition at periodontal affected sites the GCF contains considerable amounts of low molecular weight HA (Nakatani et al. 2009). Taken together, it seems plausible that these HA fragments at inflamed periodontal sites might interfere with the healing and regenerative capacity of periodontal tissues.

### 1.4.4 Cementogenic marker CAP and CEMP1

Periodontal attachment recover is primary for periodontal regeneration. Cementum protein 1 (CEMP1), as well as cementum attachment protein (CAP) are periodontal attachment related markers (Arzate et al. 2015). CEMP1 can induce cementoblasts phenotype and reduce osteoblast differentiation in PDL (Komaki et al. 2012). Both, normal human PDL cells and human immortal PDL-derived cell lines can express CAP and CEMP1 and have cementogenic potential (Torii et al. 2015). hMSCs, as mesenchymal stem cells, also have been proposed to have cementogenic

potential. However osteogenic stimulation seem to inhibit CAP and CEMP1 expression of PDL cells. On the contrary the presence of vitamin C (VC) can reverse this inhibitory effect and enhance cementogenic differentiation (Gauthier et al. 2017).

GAGs, especially HA, exist in cementum-dentin junction (CDJ) and were found important for cementum formation and mineralization (Cheng et al. 1999, Yamamoto et al. 2004, Ho et al. 2005). HA/CD44 pathway was found essential for fibroblast growth factor 2 (FGF2) in PDL cells migration (Shimabukuro et al. 2011). FGF2 induced PDL stem cells into ligamentogenic differentiation but suppress osteogenic and cementogenic differentiation (Hyun et al. 2017). It would be interesting to see if HA can stimulate whether osteogenesis and cementogenesis or ligamentogenesis.

### 1.4.5 Osteogenic and ligamentogenic markers

Alveolar bone recognition is a symbol of periodontitis convalescence. Osteoblasts secrete matrix proteins and format new bone. Therefore, osteoblast-related markers ALP, BSP, collagen type I alpha 1 (COL1A1) and OCN were chosen in this study (Weinreb et al. 1990, Karsenty et al. 1995).

ALP, BSP and COL1A1 were reported as early stage markers of osteoblastic differentiation while OCN is a late stage marker (Weinreb et al. 1990, Kuo et al. 2017). The up regulation of ALP expression reflected the rate of hMSCs committed differentiation (Jaiswal et al. 1997, Kuo et al. 2017). BSP is a phosphorylated glycoprotein which contributes to bone, dentin, cementum mineralization. It also has angiogenic capacity and gathers near primary bone (Fisher et al. 1990, Ogata 2008,

Bouleftour et al. 2016). ALP and BSP expressions were different among various donors of hMSCs (Phinney et al. 1999). For human PDL cells, ALP expression was measurable by 14$^{th}$ day of osteogenic culture while human bone marrow stem cells (hBMSCs) by 7$^{th}$ day; BSP expression was measurable both in human PDL cells and hBMSCs by 7$^{th}$ day. At day 21, collagen II and glycosaminoglycans was detected in both cells under chondrogenic induction (Gay et al. 2007).

Type I collagen is abundant in the matrix of bone, dermis, tendons and is synthesized by both osteoblast and fibroblast. COL1A1 is the most produced polypeptide chains of type I collagen so it was chosen in this research (Karsenty et al. 1995, Ghosh 2002). OCN is a small protein produced by osteoblast, odontoblasts and hypertrophic chondrocytes. It is correlated with bone mineralization, metabolism and formation (Hauschka et al. 1989, Lee et al. 2007). hBMSCs' osteogenic differentiation was improved by HA hydrogel, manifested as increased ALP, OCN, COL1A1 expressions and calcium contents (Jung et al. 2018).

Scleraxis (SCX) is the ligamentogenic marker produced mostly by osteoblast and is essential in tendon wound healing (Sakabe et al. 2018). PDL stem cells were reported to be able to express certain amount of SCX like keratinocytes. The same for COL1A1 expression (Chen et al. 2018). hMSCs, as multipotent cells, can also express SCX and develop tenogenic differentiation. SCX played an essential part in tendon differentiation progenitor of hMSCs (Alberton et al. 2012).

Former studies indicate that HMW-HA can increase proliferation and COL1A1 expression of human rotator cuff tendon derived cells (Osti et al. 2015). What HA will

militate PDL cells and hMSCs remains subject of further research.

In general, HA is rich in ECM and takes part in many cellular activities such as cell migration, proliferation and differentiation. HA also contributes to anti-inflammation, wound healing and can be used in osteoarthritis, cartilage repair, tendon healing, annulus fibrous defect, and skin repair (Wang et al. 2007, Ryan et al. 2015, Fuller et al. 2016, Ferrero et al. 2018, Piuzzi et al. 2018). How HA may affect periodontal cells yet remains unclear. It would be very interesting to see if HA can be used as a novel periodontal regeneration accelerant. To verify this hypothesis, periodontal cells hMSCs and PDL cells were chosen. As mentioned above, they both can induce osteogenesis, ligamentogenesis and cementogenesis, which means they have periodontal regenerative potential. So we used HA to stimulate hMSCs and PDL cells, then test osteogenic, ligamentogenic and cementogenic related gene expressions to see how HA will promote periodontal regeneration.

## 1.5 Aim of the study

This study aims to figure out whether LMW-HA can promote periodontal regeneration. For this purpose, the effects of LMW-HA on the cementogenic, ligamentogenic and osteogenic differentiation of periodontal cells (hMSCs and PDL cells) were explored.

# 2. Materials and Methods

## 2.1 Cell culture

### 2.1.1 hMSC

hMSCs were obtained from Lonza company (Verviers, Belgium), donated by a male caucasian and marked as 'donor VII'. Cell culture medium was α-minimum essential medium (α-MEM) (gibco-Thermo Fisher, Waltham, USA), with additive 10 % Fetal Bovine Serum (FBS) (Sigma Aldrich, Munich, Germany) and 1% Penicillin (Sigma Aldrich, Munich, Germany). Because the differentiation potential of hMSC will drop from the 6th passage on (Bonab et al. 2006), $5^{th}$ and $6^{th}$ passages of hMSCs were used in this study.

### 2.1.2 PDLhTERT

PDLhTERTs were obtained from Professor Docheva (University of Regensburg, Germany) (Docheva et al. 2010). Culture medium was high glucose-Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Munich, Germany), with additive 10 % FBS and 1% Penicillin. $28^{th}$ and $29^{th}$ passages of PDLhTERT cells were used in this experiment.

Both hMSCs and PDLhTERTs were cultured in 37℃, 5% $CO_2$, humid incubator (ThermoFisher, Waltham, USA). The culture medium was always pre-warmed to

37℃ before use and changed twice a week. Cells in early passage were firstly cultured in T75 flasks (Greiner Bio-one, Frickenhausen, Germany), then counted and seeded in different cell culture plates or flasks as required. During cell culture, when the cells got confluent, they were passed. Cells were incubated in 37℃, 5% $CO_2$, humid incubator with trypsin (Merck, Munich, Germany) for 5min, then checked under a microscope (Carl Zeiss, Jena, Germany) to see if all the cells were detached. When all the cells were detached, the culture medium was added to stop reaction. Then cells were counted with hemocytometer (Abcam, Cambridge, GB) and centrifuged with 500 rpm for 5 min under room temperature in the centrifugal machine (Thermo Fisher, Waltham, USA). The upper liquid were aspirated and then cells were diluted with acquired amount of fresh culture medium. They were then mixed well and seeded in new flasks.

### 2.1.3 Osteogenic medium and HA working medium prepare

Self-made osteogenic medium (OS), components presented as in Table 1, was used to introduce osteogenic differentiation. Dexamethasone, β-Glycerophosphate and L-Ascorbic acid (all from Sigma Aldrich, Munich, Germany) were diluted and sterilized with 0.2 μm sterile syringe filter (VWR, Radnor, USA) before use. Each time the OS medium was freshly made and discarded after 4 weeks. OS medium was kept in 4 ℃ fridge and warmed in 37 ℃ water bath before use.

To figure out the function of different molecular weight LMW-HA (Stern, Asari et al. 2006, Kavasi, Berdiaki et al. 2017), oligosaccharide nano HA (HYALOSE, Austin,

USA) and 150kDa HA (HYALOSE, Austin, USA) were chosen. HA powders, 1 mg per vial, were obtained from the company HYALOSE. Firstly they were diluted into 1 mg/ml with Dulbecco's Phosphate Buffered Saline (DPBS) (sigma-Aldrich, Steinheim, Germany). Then they were periodically vibrated in 4 ℃ for 2h to achieve complete dissolution. Diluted HA was kept in -20 ℃ fridge. To avoid multiple freeze and thaw, HA solution was aliquoted into 100 µl each eppendorf tube (Merck, Munich, Germany). Considering the HA concentrations in former studies, the working concentration 20 ng/ml was chosen in this study (Kaneko et al. 2015, Zhao et al. 2015). OS medium or DMEM was firstly warmed in 37 ℃ water bath, then added acquired amount of HA solution and mixed well. HA was unsterilized when obtained from the manufacturer. Therefore, the working HA medium was filter sterilized with 0.2 µm sterile syringe filter (VWR, Radnor, USA). Because of the filter loss, each time and each group received an extra 0.5ml of working medium. To avoid HA degradation, each time working medium was freshly made (Pigman et al. 1961).

Table 1 Constitute of Osteogenic medium.

Table 1 Constitute of Osteogenic medium.

| Osteogenic medium | Total 250ml |
|---|---|
| DMEM (Sigma Aldrich, Munich, Germany) | 219.5 ml |
| FBS (Sigma Aldrich, Munich, Germany) | 25.0 ml |
| Penicillin-Streptomycin (Sigma Aldrich, Munich, Germany) | 2.5 ml |
| Dexamethasone (Sigma Aldrich, Munich, Germany) | 0.5 ml |
| β-Glycerophosphate (Sigma Aldrich, Munich, Germany) | 1.5 ml |
| L-Ascorbic acid (Sigma Aldrich, Munich, Germany) | 1.0 ml |

## 2.2 Flow cytometry analysis

PDLhTERTs and hMSCs were divided into 6 groups, PDLhTERT groups: control, nano HA and 150k HA; hMSC groups: control, nano HA and 150k HA. Each group

was seeded in 2 flasks as duplicates. Cells were stimulated for 7 days (change medium twice) then harvested for test. On the 5$^{th}$ day of cell culture, 2 days before the harvest day, an extra control group, day 0 was seeded. Cells were detached with Accutase (Sigma-Aldrich; 5 min at 37°C) and then incubated (30 min on ice) with the following antibodies: mouse anti-human CD44-FITC and CD90-PECy7 (both BioLegend) or rabbit anti-human CD168 (Abcam). CD90 is used as a surrogate marker for stem cells. A secondary Alexa Fluor 647 donkey anti-rabbit antibody (BioLegend) was used for fluorescence detection of CD168. Sample tubes were acquired on a BD FACSAria III (BD Biosciences) and 10.000 gated events were recorded. Data were analyzed with BD FACSDiva and FlowJo V10 software.

## 2.3 Immunofluorescence analysis

Immunofluorescence is the technique that made antibodies bond to the specific epitope of the antigen within the cells visual. CD44, CD168 and TLR4 antibodies were chosen in this research. hMSCs and PDLhTERTs were made slides, stained with these antibodies and made immunofluorescence images.

### 2.3.1 Slides preparation

hMSCs and PDLhTERTs were divided into 6 groups, hMSC groups: control, nano HA and 150k HA; PDLhTERT groups: control, nano HA and 150k HA.

Cells were seeded on glass slides (Menzel, Munich, Germany): 0.5ml per slides, 40,000 cell/ml. Slides were laid into quadrilPERM dishes (Sarstedt, Nümbrecht,

Germany) and incubated for 2h in 5% $CO_2$, 37℃ incubator to get the cells attached. Slides were then checked under a microscope (Carl Zeiss, Jena, Germany) to ensure that all cells were attached. Then 2 ml working medium was added into each dish as designed and cells were incubated overnight. On the next day, slides were fixed with methanol (Carl Roth, Karlsruhe, Germany) and kept in -20℃ fridge before staining.

### 2.3.2 Staining with CD44 and CD168 antibodies

Before staining, slides were divided into 3 parts with wax pen: control part, 1:50 and 1:100 dilutions of antibody parts.

Firstly, slides were washed 5min with washing buffer: phosphate-buffered saline (PBS) (Sigma Aldrich, Munich, Germany) with 1% Tween-20 (Carl Roth, Karlsruhe, Germany). Then slides were blocked with 10 % horse serum in dark ( 1 h at room temperature). After that, control groups were incubated with PBS; other groups incubated with diluted primary antibodies: CD44, CD168 (Santa Cruz, Heidelberg, Germany). Then slides were incubated in 4 °C fridge in dark overnight. On the next day, the slides were treated with 1:500 AlexaFluor 488 anti-rabbit and anti-goat IgG medium (Invitrogen, Eugene, OR, USA) in room temperature for 1 hour, and then washed with washing buffer. After that all slides were applied with 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen, Eugene, OR, USA) with concentration of 1:10000 for 1min, then washed again. All the solutions used in this experiment, such as washing buffer, antibody solutions, DAPI solution and so on, were freshly made.

### 2.3.3 Seal slides with cover glass

After staining, all slides were sealed with ProLong™ Gold antifade reagent (ProLong™ , Eugene, USA) with 24*50mm cover glass (Menzel, Munich, Germany). Bubbles were pressed out until at least working parts of the slides were clear. Slides were then kept in 4℃ in dark.

### 2.3.4 LSM510 confocal imaging

Images were obtained with Laser Scanning Microscope 510 (LSM 510) and AxioCam MRc (both Carl Zeiss, Jena, Germany). Confocal channels DAPI and Alexa 488 were chosen in the program Axio Vision (Carl Zeiss, Jena, Germany). DAPI filter was used to obtain the core images of the cells and 44FITC filter to obtain the cytoplasm images, both under x63 oil ocular. All the images were obtained within 1 week after staining.

## 2.4 Polymerase chain reaction

This study aims to figure out the effects of LMW-HA on the cementogenic, ligamentogenic and osteogenic differentiation of human hMSCs and PDL-hTERTs. To do PCR needs cDNA of the cells and related primers. Cementogenic related primers, ligamentogenic related primers and HA related primers were self-designed. Osteogenic related primers were acquired from company. They were all proved viable with their specific positive controls.

hMSCs and PDL-hTERTs were divided into 6 groups, hMSC groups: control, +OS+nano HA and +OS+150k HA; PDLhTERT groups: control, +OS+nano HA and +OS+150k HA. They were cultured for 0, 3, 7, 21 days with working medium as designed and harvested by different time points. Harvested cells were firstly isolated RNA and then transferred into cDNA for PCR tests.

## 2.4.1 cDNA prepare

### 2.4.1.1 Cell culture and sample harvest

hMSCs in the 5$^{th}$ passage and PDLhTERTs in 27$^{th}$ passage were firstly cultured in T75 flasks. When they were confluent, cells were treated with trypsin (Merck, Munich, Germany). When all the cells were detached, they were added with culture medium to stop reaction and then counted. About 1 million hMSCs and PDLhTERTs were collected into two separate tubes (Merck, Munich, Germany), which were considered as day 0, the starting line. The rest of the cells were seeded in T25 flasks and divided into 8 groups, hMSC groups: control; +OS; +OS+nano HA; +OS+150k HA; PDLhTERT groups: control; +OS; +OS+nano HA; +OS+150k HA. After 48h, when all cells were attached, they were changed with working medium and stimulation started. Time points were designed as 3 day, 7 day, and 21 day.

Cells of different time points were harvested separately with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Firstly the upper liquid of cells were aspired, then required amount of TRIzol reagent was added. All the flasks were then put on ice, later operations were all done on ice. Cells were scraped (Greiner bio-one,

Frickenhausen, Germany) and collected in different labeled tubes (Merck, Munich, Germany). Samples were stored in -80℃ fridge for RNA isolation.

#### 2.4.1.2 RNA isolation

Samples with TRIzol reagent were thawed at room temperature. Then chloroform (Sigma Aldrich, Munich, Germany) was added (200 µl chloroform for 1ml TRIzol) and mixed well. Then all the samples were centrifuged 10,000 rpm for 15 min in a centrifuge (Thermo Fisher, Waltham, USA). After centrifugation, liquid was divided into different layers. The upper transparent layer was carefully collected into RNA-free eppendorf tubes (Merck, Munich, Germany). The volume of the collected upper transparent liquids were measured. Same volume of 70% ethyl alcohol (Sigma, St. Louis, USA) was added into tubes and mixed gently.

RNeasy Mini Kit 250 (Qiagen, Hilden, Germany) was used for RNA isolation. Each time a maximum of 700 µl liquid (made last step) was added into the rose tube from the kit. Rose tubes were centrifuged 10000 rpm for 15s, then added 350 µl RW1 and centrifuged again. DNase mix, 10 µl DNase diluted in 70 µl RDD Buffer for each sample, was counted and made. DNase and RDD Buffer were obtained from RNA-free DNase set 50 (Qiagen, Hilden, Germany). Then 80 µl DNase mix was added into each rose tube. Rose tubes were incubated (15 min, room temperature) and washed with 350 µl RW1 again. Then 500 µl RPE Buffer was added into each rose tubes and centrifuged 10000 rpm for 1min. Until now the liquids after centrifugation of all the former steps were discarded. The rose tubes were then changed with new

RNA-free 2ml tubes and added 50 µl RNA-free water. Tubes were incubated (1 min, room temperature) and then centrifuged 10000 rpm for 1 min. What left in the tubes now was sample RNA.

All RNA samples were tested with the NanoDrop™ machine (Thermo Fisher, Waltham, USA) for optical density A260/A280 ratio. Samples' inclusion criteria was A260/A280 ratio from 1.8 to 2.1. All sample RNA were stored in -80℃ fridge.

#### 2.4.1.3 RNA transferred to cDNA

To unify the start line of PCR, cDNA amount of different samples should be the same. Thus for each sample the total amount of RNA for cDNA transfer should also be the same. Due to biologic differences and other effect factors the concentration of isolated RNA were not the same, even in the same group of same cell type. Therefore the volume of RNA used should be calculated as following.

The total amount of RNA was settled as 500 ng per sample and the total volume of each sample (RNA+H2O) was settled as 11 µl. The calculation equation was as follows and an example is presented as in Table 2. RNA concentration between 45.5 (ng/µl) and 500 (ng/µl) can be calculated like this. For those samples with RNA concentration lower than 45.5 (ng/µl), they were heated till all water evaporated and then diluted to the needed concentration. For those samples with RNA concentration over 500 (ng/µl), they were diluted to lower concentrations and then calculated as normal.

RNA calculation equation:

500ng RNA needed RNA sample volume (µl) = 500 ng / RNA concentration (ng/µl)

needed $H_2O$ (µl) = 11 (µl) - 500ng need RNA (µl)

Table 2 RNA calculation.

| Sample | RNA concentration (ng/µl) | 260/280 | 260/230 | 500ng need RNA (µl) | need $H_2O$ (µl) | Total volume (µl) |
|---|---|---|---|---|---|---|
| NO.1 | 53,31 | 2,01 | 1,65 | 9,38 | 1,62 | 11,00 |
| NO.2 | 141,94 | 2,05 | 1,03 | 3,52 | 7,48 | 11,00 |

All diluted sample RNA should be denatured before transfer, procedure was as in Table 3. When denaturing finished, sample probes were put on ice immediately and started RNA transfer to cDNA . All the pipetting work was done on ice with materials obtained from first-strand cDNA Synthesis Kit (Roche, Mannheim, Germany),

presented in Table 4. Filtered tips were changed every time to avoid contamination.

To assure no contamination during transfer procedures, negative controls were made. Two extra tubes labeled ‘neg 1’ and ‘neg 2’ were prepared. For these two negative controls, PCR grade $H_2O$ (SG, Roche, Mannheim, Germany) was used as replacement of sample RNA added into probes. Except that, other steps were the same as RNA samples. In RNA transfer to cDNA procedure, as showed in Table 4, ‘neg 1’ was lack of AMV Reverse Transcriptase and ‘neg 2’ not. Apart from that difference, negative controls were handled as other samples.

Table 3 Denaturing procedure.

| Reaction-Mix | |
|---|---|
| Probe (RNA+ $H_2O$) | 11.0 µl |
| Hexamer Primer | 2.0 µl |
| Procedure: | |
| 1=65℃ for 15min | |
| 2=4℃ ∞ | |
| 3=end | |

Then RNA was to cDNA. The procedure was as following.

Table 4 RNA transfer to cDNA procedure

| Reaction Mix 20.0 µl in total | |
|---|---|
| Rxnbuffer | 4.0 µl |
| dNTPs (10 mM each) | 2.0 µl |
| RNAse Inhibitor 40U | 0.5 µl |
| AMV Reverse Transcriptase | 0.5 µl |
| Probe (RNA+ $H_2O$+ Hexamer Primer) | 13.0 µl |

Procedure:

1=25℃ for 15min

2=50℃ for 60min

3=85℃ for 5min

4=4℃ for 5min

5=4℃ ∞

6=end

cDNA samples, including negative controls (neg1 and neg 2), were stored in -20℃ fridge for future PCR test.

## 2.4.2 Self-designed primers

Primers: CD44, CD168, TRL-4, CAP, CEMP1, ALP, BSP, COL1A1, OCN, and SCX were tested in this research. Of these 10 primers, CD44, CD168, TLR4, CAP and CEMP1 were self-designed. SCX sequences was obtained from literature (Schulze-Tanzil et al. 2004).

### 2.4.2.1 Primer sequence design

Pubmed was used for self-designed primers. For example, inquire 'Homo sapiens; CD44 mRNA' in Pubmed and a series of primer pairs were obtained, as shown in Table 4 and 5. For one primer about 4 different primer pairs were obtained from Pubmed gene bank for test.

The viability and annealing conditions of these 6 primers were tested by PCR and verified in gel electrophoresis. If primer pairs were not working, new primer pairs were designed and tested again until viable primer was found. All these primers were provided by TIB-MOLBIOL (Berlin, Germany).

Table 5 CD44 Primer pair 1.

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|

Table 5 CD44 Primer pair 1.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | AGAAGAAAGCCAGTGCGTCTC | Plus | 21 | 2 | 22 | 61,08 | 52,38 | 3,00 | 3,00 |
| Reverse primer | TGCTCTGCTGAGGCTGTAAAT | Minus | 21 | 129 | 109 | 60,17 | 47,62 | 6,00 | 2,00 |
| Product length | 128 | | | | | | | | |

Table 6 CD44 Primer pair 2.

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC % | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | GGCAGCCCCGATTATTTA | Plus | 18 | 96 | 113 | 58,52 | 50,00 | 5,00 | 2,00 |
| Reverse primer | GCTGCAGTTTTTATTCGAGGT | Minus | 21 | 285 | 265 | 58,53 | 42,86 | 6,00 | 0,00 |
| Product length | 190 | | | | | | | | |

#### 2.4.2.2 Viability test of self-designed primers, PCR part

To verify primer is viable or not needs to test with positive controls. For one primer 4 possible positive controls were used. PDL and hMSC were used for main purpose of this research so they both were chosen as positive controls. Other positive controls were found in protein atlas. Positive controls tested for each primer were presented as in Table 6. Positive controls were made cDNA and stored in -20℃ fridge for future test.

Table 7 Positive control of self-designed primers.

| Primer | Positive control | | | |
|---|---|---|---|---|
| CD44 | PDL cell | hMSC | Bone | Teeth |
| CD168 | PDL cell | hMSC | Bone | Teeth |
| TLR4 | PDL cell | hMSC | Bone | Colon tissue |
| CAP | PDL cell | hMSC | Bone | Caco-2 |
| CEMP1 | PDL cell | hMSC | Bone | Caco-2 |
| SCX | PDL cell | hMSC | Bone | Tendon |

PCR was used to verify primer works with positive control. PCR master mix was prepared as follows: A sterile 1.5 ml RNA-free eppendorf tube (Merck, Munich, Germany) labeled as 'master mix' was prepared. It was then pipetted into appropriate amount of primer, $H_2O$, and Syber Green Master I (Roche, Mannheim, Germany) as presented in Table 7, then mixed well. 15 µl of the master mix was pipetted into RNA-free eppendorf tubes and then 5 µl of positive control cDNAs. When finishing pipetting the tubes were mixed well and centrifuged to make sure all liquids were in the bottom of the tubes without bubbles. Then the tubes were run in Dyad Peltier Thermal Cycler machine (San Diego, USA) under specific thermocycling conditions.

The appropriate thermocycling condition, especially annealing temperature of self-designed primers were unknown. To figure out the thermocycling conditions of these primers, several tests were done. Pubmed gene bank has already provided a suggested annealing temperature for each primer pair. We settled the running annealing temperature 1 or 2 degrees higher and lower than the suggested annealing temperature to find the most appropriate one. For example, the suggested annealing temperature of CD44 pair 1 was 60℃, so the testing annealing temperatures of CD44 pair 1 was 59℃ and 61℃. Then PCR of different annealing temperatures were run in PCR, then PCR products were used for gel electrophoresis. Primer pair of the best

stain in gel electrophoresis image was chosen. Same procedures were done for all the primer pairs with different annealing temperatures. If the normal PCR results were unsatisfactory, touch down PCR was applied.

Table 8 PCR Master Mix and thermocycling condition

| | | |
|---|---|---|
| Primer | 2.0 µl | Master Mix: 15.0 µl |
| $H_2O$ | 3.0 µl | |
| Syber Green I Master | 10.0 µl | |

PCR Procedure:

1=95℃ for 15min

2=94℃ for 30sec

3=Annealing temperature for 30sec

4=72℃ for 60sec

5=72℃ for 10min

6=end x 45 cycles

### 2.4.2.3 Viability test of self-designed primers, gel electrophoresis part

PCR products were used for gel electrophoresis and then digital images were obtained. Depending on the digital images, the most appropriate primer pairs and thermocycling conditions were chosen.

Gel making

Gel electrophoresis can be used for analysis of DNA, RNA and proteins by their size and charge. First of all an agarose gel was made. 1.8 g agarose (Biozym, Hessisch Oldendorf, Germany ) was diluted in 100 ml Tris-borate-EDTA (TBE) buffer (Thermo Fisher, Waltham, USA) in a glass bottle. Then 4 µl Ethidium Bromide (EB) (Apotheke Klinikum Innenstadt, Munich, Germany) was added into the bottle. Because the toxicity of EB, separate EB only instruments and working place were required. Operator must wear glove during the whole procedure working with EB. To help agarose dilute the whole bottle was heated 3 times in microwave oven shortly. When the agarose was completely diluted and no macroscopic bubbles inside, the solution was poured into the plate. A comb was inset into the gel quickly. The gel was checked carefully to make sure no bubbles were in working part. If there was a bubble, it should be removed quickly while the agarose gel was still hot. About 30 min later the gel was cooled down and became solid. The comb was then removed and the gel was ready to use.

Electrophoresis running

A gel electrophoresis apparatus (Bio-rad, Munich, Germany) was prepared. Gel made as described previously was put into the box filled with TBE buffer. PCR products (20 µl) were mixed with 3 µl DNA application buffer orange G sodium (Apotheke Klinikum Innenstadt, Munich, Germany). DNA ladder and PCR product mix were loaded into the slots made by the comb, each slot 5 µl. They were all loaded gently into the bottom of the slot first, then step back slowly to make sure all the sample was inside the slot. Then gel electrophoresis apparatus was connected to electric power. The negative terminal (black wire) was connected with the slots side and the positive terminal (red wire) was connected with the other side of the gel. Running was started at 80V for 15 min, then at 100V for about 30 min, which depended on actual situation.

The extent of running was judged by orange G sodium, which was orange color and can be seen in the gel. The perfect running time is to let the orange G sodium run to about 2/3 length of the gel. If running too long, the PCR products may run out of the gel, which may lead to the failure of the test. If running was too short, the DNA ladder may be not long enough to separate different molecular markers, which may cause difficulties in differentiation between target primer and the ladder.

Digital image taking

When gel electrophoresis was finished, digital images were made with Peqlab machine (Erlangen, Germany). As we can see in the following CD44 pair 1 gel

electrophoresis image Figure 1, compared with DNA ladder (the brightest stain was 100 bp), stain of hMSCs were at the right place (CD44 pair 1 was 128 bp). Of all 4 positive controls hMSC stains were positive. Annealing temperature 61℃ was brighter than the stain of 59 ℃ annealing temperature. Other primer pairs of CD44 were not viable and showed no reasonable staining on the images. In summary, hMSC was chosen as positive control for viable primer CD44 pair 1 and the thermocycling protocol was also settled as tested.

Same procedures were applied for CD168, TLR4, CAP, CEMP1 and SCX (Table 8). For SCX one primer pair was obtained from literature, another 3 primer pairs were self-designed as mentioned above. Finally the sequences obtained from literature were chosen.

Also for the housekeeping gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH), the primer was self-designed as done for the other genes (TIB-MOLBIOL, Berlin, Germany) and tested to make sure its practicable. Housekeeping gene means it is expressed in allmost every kind of cell type under any normal thermocycling condition. Therefore any cell type can be used as GAPDH positive control and in this study PDL cell' cDNA was chosen and worked well. Also GAPDH is expressed under any normal thermocycling condition. In this study GAPDH was run under 3 different thermocycling conditions and the primer was controlled if the thermocycling conditions are appropriate for it.

Figure 1 CD44 pair 1 gel electrophoresis result-1.

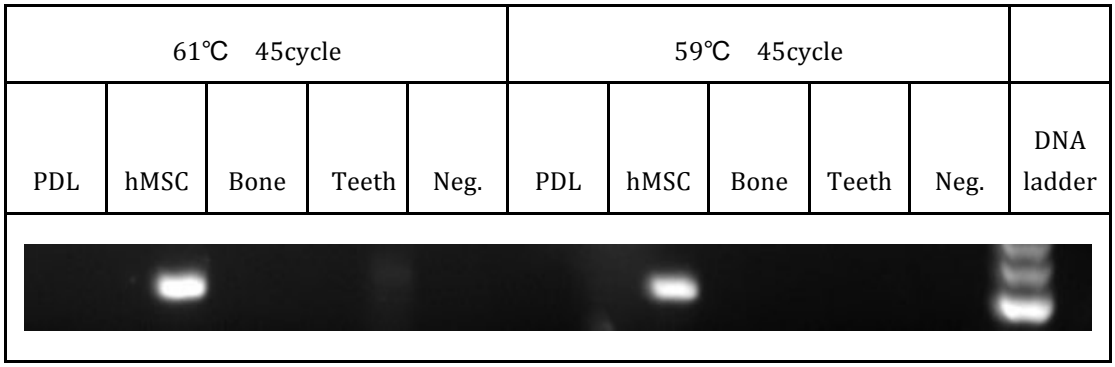


Figure 1 CD44 pair 1 gel electrophoresis result. In DNA ladder the brightest stain was 100bp. CD44 pair 1 was 128 bp. Stain of annealing temperature 61℃ was brighter than 59℃ in hMSC cDNA. Neg. was negative control.

Table 9 Primer sequences, positive controls and thermocycling conditions

| Primer | Sequence 5'-3' | Positive control | Annealing condition | Length (bp) |
|---|---|---|---|---|
| GAPDH | for: CAA CTA CAT GGT TTA CAT GTT C<br>rev: GCC AGT GGA CTC CAC GAC | PDL | 61℃/65℃ x45cy<br>or<br>Touch down<br>68-58℃x45cy | 181 |

Table 9 Primer sequences, positive controls and thermocycling conditions

| | | | | |
|---|---|---|---|---|
| CD44 | for: AGA AGA AAG CCA GTG CGT CTC<br>rev: TGC TCT GCT GAG GCT GTA AAT | hMSC | 61℃x45cy | 128 |
| CD168 | for: AGT CTT CGG AAT CAA AGG AAT CT<br>rev: GCA TTT AGC CTT GCT TCC ATC | hMSC | 61℃x45cy | 154 |
| TLR4 | for: CAG CTC TTG GTG GAA GTT GA<br>rev: GCA AGA AGC ATC AGG TGA AA | Colon tissue | Touch down 68-58℃x45cy | 191 |
| CAP | for: GGG GTC CAA GTG AGT TCA AGA<br>rev: AAC CCA ACT CCT TTT TGT CCA | Caco-2 | 61℃x45cy | 183 |
| CEMP1 | for: TCA AGA CAA TCA CCC CTG AC<br>rev: AAC CCT ATC TCT TCA CAC ATC C | Caco-2 | 65℃x45cy | 299 |
| SCX | for: CCT GAA CAT CTG GGA AAT TTA ATT TTA C<br>rev: CGC CAA GGC ACC TCC TT | tendon | Touch down 68-60℃x45cy | 111 |

### 2.4.3 Osteogenic related primers

Osteogenic related primers were ALP, BSP, COL1A1 and OCN. They were provided directly by LightCycler primer set (Roche, Heidelberg, Germany). Primer set included primer, positive control, standard and standard stabilizer. Since they were provided by the company, viability test was done together with standard curve test

which will be introduced in detail in the following text. Thermocycling conditions of these primers are listed in Table 10. Primer sequence, product size, standard and positive control were trade secrets and were not provided by the company. GAPDH for OS markers was also obtained from LightCycler primer set (Roche, Heidelberg, Germany) and thermocycling condition was the same.

Table 10 Thermocycling conditions of osteogenic related primers.

| Primer | Thermocycling condition |
|---|---|
| ALP | Touch down 68-60℃x45cy |
| BSP | Touch down 68-60℃x45cy |
| COL1A1 | Touch down 68-60℃x45cy |
| OCN | Touch down 68-60℃x45cy |

## 2.4.4 rt-qPCR

All the samples (cDNA) were tested with quantitative real time polymerase chain reaction (rt-qPCR) machine LightCycler 480 (Roche, Mannheim, Germany). For nucleic acid stain, SYBR Green I Master (Roche, Mannheim, Germany) was used.

PCR grade $H_2O$ (Roche, Mannheim, Germany) was used during the entire process.

Firstly, a standard curve for each primer was made. Because self-designed primers and osteogenic related markers were obtained form different sources these two kinds of primers were operated differently in standard curve making.

cDNA samples were run with rt-qPCR and the CP values were detected. Gene expressions were counted according to their standard curve. Melting curves of each sample was checked, those values with wrong melting curve were deleted. Finally the relative expression was normalized against GAPDH as housekeeping gene.

#### 2.4.4.1 Standard curve of self-designed primer

For self-designed primers, only the primer was provided by the company. The positive control tested before was used as standard. PCR grade $H_2O$ (Roche, Mannheim, Germany) was used as standard stabilizer.

Standard dilution

Firstly, 6 sterile 1.5 ml RNA-free eppendorf tubes (Merck, Munich, Germany) labeled with 'STD 1:2'; 'STD 1:4'; 'STD 1:8', STD 1:16'; 'STD 1:32'; 'STD 1:64' were prepared. Then 30 µl CR grade $H_2O$ was pipetted into each tube. After that 30 µl cDNA of positive control of the primer was pipetted into the 'STD 1:2' tube, centrifuged and then mixed well. Then 30 µl 'STD 1:2' was pipetted into 'STD 1:4' tube, centrifuged and mixed as former step; other dilutions followed the same procedure. A separate tube of undiluted positive control cDNA was labeled with 'STD

1:1' and used for standard curve only. All the tips used for PCR were RNA-free and with filter. Tips were changed every time to avoid contamination.

rt-qPCR

A sterile 1.5 ml tube labeled as 'PCR Master Mix' was prepared. Appropriate amount of primer, $H_2O$, and Syber Green I Master as presented in Table 11 were pipetted in and mixed well. Sample cDNAs were diluted into 1:20 (190 µl $H_2O$ and 10 µl cDNA). 15 µl of the PCR Mix were pipetted into a 384-well PCR plate (Roche, Mannheim, Germany) and then 5 µl of diluted cDNA (1:20) were added as well. When pipetting was finished the plate was sealed with a special PCR parafilm (Roche, Mannheim, Germany). Then the plate was centrifuged 1500 rpm for 2 min to make sure all liquids were in the bottom of the wells and without bubbles. After that the plate was tested with rt-qPCR machine LightCycler 480 (Roche, Mannheim, Germany) with specific thermocycling conditions of different primers.

Table 11 PCR master mix preparation

| | | |
|---|---|---|
| Primer | 2.0 µl | Master Mix for one sample: 15.0 µl |
| $H_2O$ | 3.0 µl | |
| Syber Green I Master | 10.0 µl | |
| Total Master Mix: | | |
| Sample number x3 + positive control x3 + negative control x3 + STD dilution number x3 + 4 extra = PCR Mix | | |

Triplicate technical repeats were made for each sample. Each technical repeat represented by green points on the standard curve image (Figure 2). Those technical repeats (green points) with extreme deviation from the standard curve were deleted. Figure 2 was an example of self-designed primer: CD44's standard curve. Other self-designed primers' standard curves were alike.

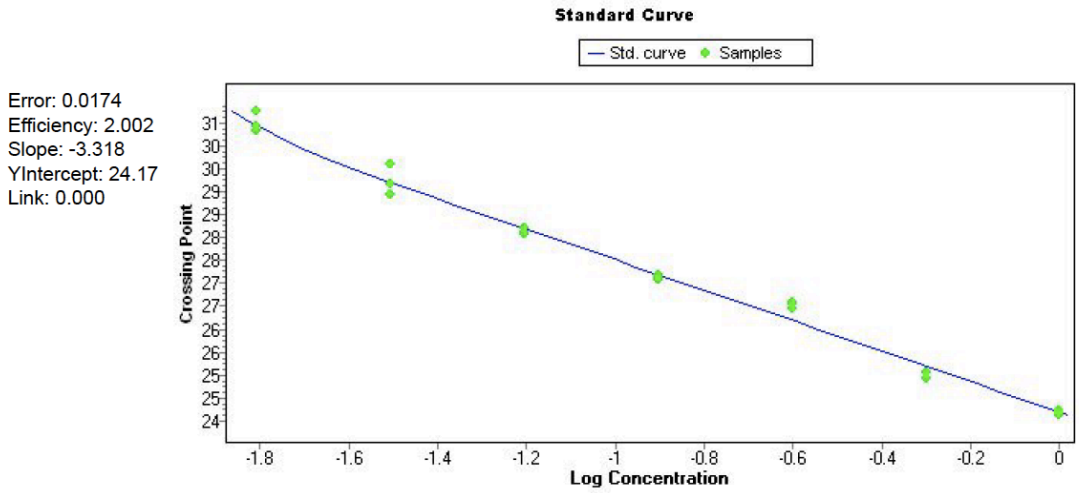


Figure 2 Standard curve of self-designed primer CD44. One colony of green points represent one standard dilution, from left to right '1:64; 1:32; 1:16; 1:8; 1:4; 1:2; 1:1'.

### 2.4.4.2 Standard curve of osteogenic primer

The procedure of making standard curve for osteogenic primers was almost the

same as with self-designed primers, except for standard dilutions. For osteogenic primers, standard, standard stabilizer, positive control and primer were all provided by the LightCycler primer set (Roche, Heidelberg, Germany).

Firstly, 3 sterile 1 ml RNA-free eppendorf tubes (Merck, Munich, Germany) labeled with 'STD 1:10'; 'STD 1:100'; 'STD 1:1000' were prepared. Then 27 µl standard stabilizer was pipetted into each tube. After that 3 µl standard was pipetted into the 'STD 1:10' tube, centrifuged and then mixed well. Then 3 µl 'STD 1:10' was pipetted into 'STD 1:100' tube, centrifuged and mixed as former step, other dilutions followed the same procedure. Liquid was pipetted by the edge of the tube and tips were changed every time to avoid contamination. Then PCR master mix was made as in Table 11 and pipetted into a 384-well PCR plate (Roche, Mannheim, Germany) following the protocol as mentioned before.

Triplicate technical repeats were made for each dilution of osteogenic primers. Figure 3 presents an example of osteogenic primer showing the standard curve for ALP. Other osteogenic primers' standard curves were alike.

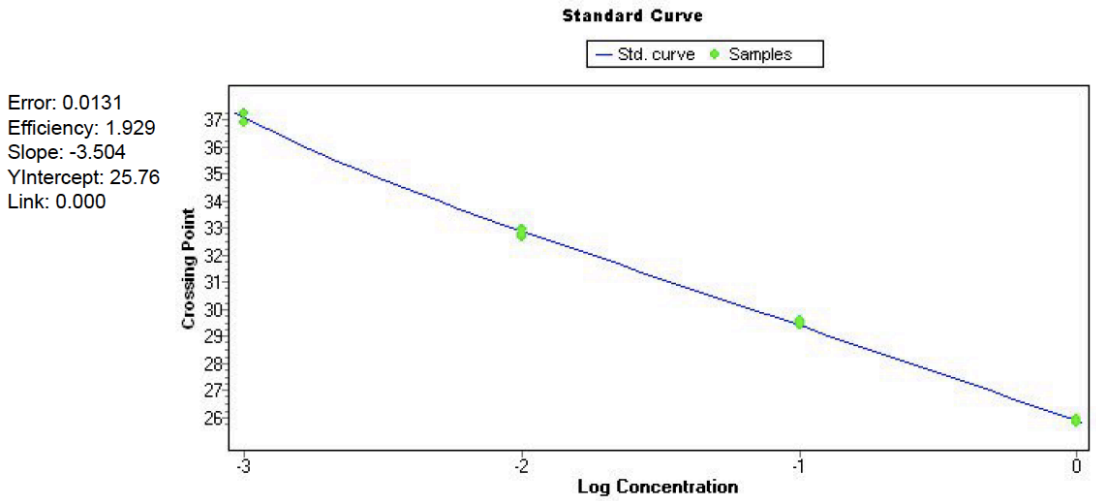


Figure 3 Standard curve of self-designed primer CD44. One colony of green points represent one dilution of the standard, from left to right : '1:1000; 1:100; 1:10 and 1:1'. Each dilution had 3 technique repeats.

### 2.4.4.3 Technical repeats of PCR run

To prove the repeatability PCRs should be run at least twice and were run at different time. Therefore technical repeats of PCR run were required. Samples from the same cDNA dilution can share the same primer standard curve. Which means, when making PCR technical repeat, one standard dilution was required to be repeated together with the samples, as showed in Figure 4. Only standard part could be simplified, other procedures of PCR technical repeats were the same as for the first run.

In one PCR run each sample was made in triplicate technical repeats, and at least one PCR technical repeat (depend on the varieties of two data). The total technical repeats of one sample was at least six times.

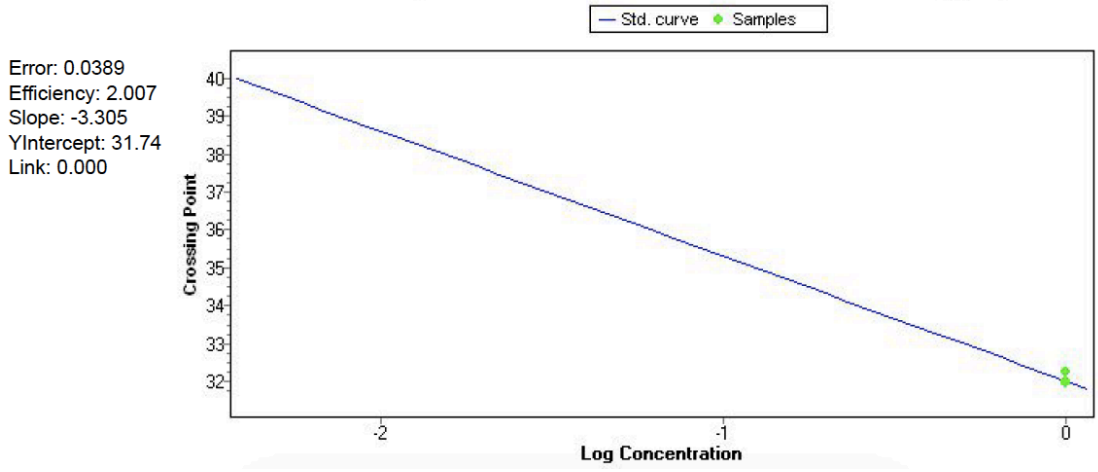


Figure 4 PCR technique repeat standard curve. Two green points were repeated standard dilution 1:1. The standard curve was made before.

### 2.4.4.4 Melting curve control

The melting peaks of the same primer for all the samples in PCR should be the same. For those values who did not share the same melting peak with others, they were not included into the final data analysis. Also, negative controls should not be in the same melting peak with the samples. As shown in Figure 5, most samples had the same melting temperature and formed the main peak; negative controls had either earlier melting peaks (two small peaks below 85 ℃) or no peaks (flat red lines beneath).

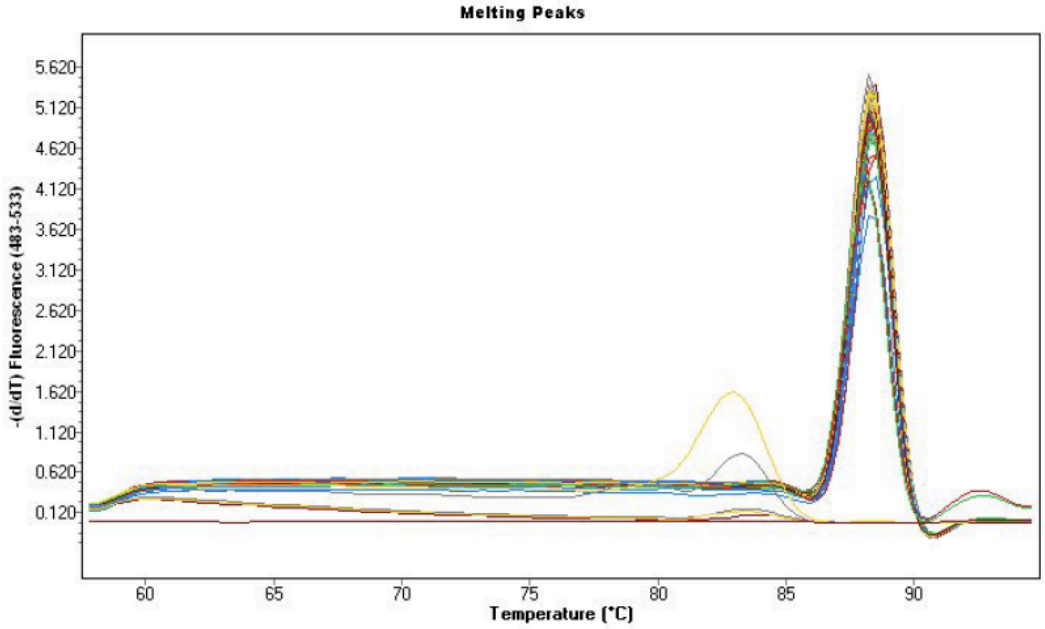


Figure 5　Melting curve of samples. Most of the samples had the same melting temperature (main peak); negative controls had either earlier melting peaks (two small peaks below 85 ℃) or no peaks (flat red lines beneath). For those values who did not share the same melting peak with others, they were not included in data analysis.

### 2.4.4.5　CP value to gene expression

CP values were detected by PCRs. Sample concentrations were calculated according to the standard curve of different primers. Due to biodiversity, sample concentrations were controlled by housekeeping gene GAPDH. Controlled gene expression were then used for data analysis. The equation was as following:

Control equation

Controlled primer expression = sample primer concentration / sample GAPDH concentration

## 2.5 Von Kossa stain

Von Kossa stain is a quantified technique that can detect the mineral deposition of the cells. Cells were fixed, stained, dipicted photographically and compared by their shade.

hMSCs and PDLhTERTs were seeded in 12-well culture plates (Greiner bio-one, Frickenhausen, Germany) separately. Cells were divided into 8 groups, hMSC groups: control, +OS, +OS+nano HA and +OS+150k HA; PDLhTERT groups: control, +OS, +OS+nano HA and +OS+150k HA. All these groups were stimulated with different working medium as designed for 21 days and made von Kossa stain timely. For von Kossa stain, cells were firstly fixed, then stained.

### 2.5.1 Cell fixing

At day 21, cells were harvested for von Kossa stain. Medium was aspirated from each well and cells were washed twice with PBS (Sigma Aldrich, Munich, Germany). Then each well was incubated with 1ml methanol (Carl Roth, Karlsruhe, Germany)

(20 min, -20℃). After that, the methanol was aspirated from each well carefully (methanol discarded in a special bottle) and washed with distilled water.

### 2.5.2 Von Kossa stain

Fixed cells were firstly incubated with 5% Silver Nitrate (Merck KGaA, Darmstadt, Germany) in the dark (10 min, room temperature, Silver Nitrate discarded in a special bottle), and then washed with distilled water. After that cells were incubated with 1% Pyrogallol acid solution (University Pharmacy, Munich, Germany) 5 min for plasma dyeing and washed again with distilled water. Then cells were incubated with sodium hydroxide solution (Merck KGaA, Darmstadt, Germany) and rinsing carefully with tape water for 15 min. To get the nuclear stained, cells were incubated with May-Grünwald solution (Merck KGaA, Darmstadt, Germany) for 10 min and then washed with distilled water twice. Distilled water used for rising were all aspirated in former steps. After the last washing, 1 ml distilled water was kept in each well.

Materials needed:

5% Silver Nitrate solution 15 ml (M=169.87 g/mol)

169.87 * 5% x 0.015L = 127.4025 mg diluted into 15ml distilled water

5% Sodium Nydroxide 15 ml (45% NaOH in stock)

5% * 15ml/ 45% = 1.67 ml diluted into 13.34ml $H_2O$

### 2.5.3 Von Kossa stain images capture

After staining, images were captured by same person with the same digital camera (Nikon 7200, Natori, Japan) at the same place under the same light to minimize varieties. The photos were adjusted into black and white to refrain from chromatic aberration.

## 2.6 Calcium deposition analysis

Calcium deposition analysis is a technique that can quantitatively detect calcium concentrations of cells. Cells were harvested and tested with enzyme-linked immunosorbent assay (ELISA). Calcium concentrations of samples were calculated according to the standard curve.

Cell culture and grouping for calcium deposition analysis were the same as used for von Kossa stain: hMSC groups: control, +OS, +OS+nano HA and +OS+150k HA; PDLhTERT groups: control, +OS, +OS+nano HA and +OS+150k HA. Cells were also stimulated for 21 days with designed working medium.

Cells were harvested with hydrochloric acid (Sigma, St. Louis, USA) and cell scrapers (Greiner bio-one, Frickenhausen, Germany). Culture medium was aspirated and each well was added into 500 μl hydrochloric acid. Then cells were scraped with cell scrapers and the turbid liquid was collected. Another 500 μl hydrochloric acid was added into each well to rinse cell fragments attached to the bottom and collected the liquid into the former tube of this well. Each well was checked under the

microscope to control whether all the cells were perfectly collected. Samples were stored in -20℃ fridge for future ELISA test.

QuantiChromTM Calcium Assay kit (Bioassay Systems, Basel, Switzerland) was used for the ELISA test to evaluate calcium concentration of samples. The kit contained standard, reagent A and reagent B. Standard dilutions were made as presented in Table 11. Total volume of each dilution was 100 µl. Standard 1 was the original standard and blank was $H_2O$. Working reagent was reagent A combined with same volume of reagent B. Each well needed 200 µl working reagent. Samples were thawed in room temperature and mixed well. 5 µl of each sample was pipetted in a 96-well flat bottom plate (Thermo Fisher, Waltham, USA) carefully. Then 200 µl regent was added into each well and incubated 3 min in room temperature. The results were all read with 612 nm wavelength in the same ELISA machine (TECAN, infinite M200, Switzerland) timely. Duplicate technical sample repeats and triplicate ELISA run repeats were made like PCR test.

**OD value to calcium concentration**

The following Table 12 was part of the raw data (OD value) of PDLhTERT calcium deposition ELISA. This is an example to explain how to calculate the calcium concentration from raw data.

Table 12 Raw data of PDLhTERT calcium deposition ELISA.

Table 12 Raw data of PDLhTERT calcium deposition ELISA.

OD value of PDL-hTERT 1 Calcium deposition (cell harvest on 22.06.16)

| | Run 1 | | | Run 2 | | | Run 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Blank | 0.6032 | 0.6044 | 0.5995 | 0.5491 | 0.546 | 0.5481 | 0.5377 | 0.5404 | 0.5343 |
| Standard1 | 1.3536 | 1.3516 | 1.3549 | 1.3274 | Missing | 1.3769 | 1.2884 | 1.3039 | 1.2393 |
| Standard2 | 1.2011 | 1.2197 | 1.2188 | 1.2439 | 1.207 | 1.2401 | 1.182 | 1.1941 | 1.1806 |
| Standard3 | 1.0851 | 1.0586 | 1.0544 | 1.0611 | 1.0824 | 1.071 | 1.0683 | 1.0362 | 1.0462 |
| Standard4 | 0.9301 | 0.9046 | 0.9282 | 0.9195 | 0.9122 | 0.9152 | 0.8886 | 0.8953 | 0.8814 |
| Standard5 | 0.8509 | 0.8539 | 0.8637 | 0.8228 | 0.8259 | 0.8304 | 0.815 | 0.8139 | 0.8079 |
| Standard6 | 0.7577 | 0.7683 | 0.7786 | 0.7398 | 0.7382 | 0.7407 | 0.7225 | 0.7281 | 0.723 |
| Standard7 | 0.6794 | 0.6747 | 0.6764 | 0.6354 | 0.6234 | 0.6369 | 0.6259 | 0.6225 | 0.6212 |
| PDL1 con1 | 0.6407 | 0.6455 | 0.6505 | 0.5951 | 0.5899 | 0.5919 | 0.5734 | 0.5716 | 0.5715 |
| PDL1 con2 | 0.626 | 0.6388 | 0.6359 | 0.5838 | 0.5839 | 0.5781 | 0.5636 | 0.5632 | 0.5612 |
| PDL1 con3 | 0.6282 | 0.6413 | 0.6437 | 0.5894 | 0.5841 | 0.5815 | 0.5641 | 0.5634 | 0.5643 |
| PDL1 OS1 | 0.7482 | 0.7654 | 0.769 | 0.7462 | 0.7274 | 0.7375 | 0.7154 | 0.7081 | 0.7065 |
| PDL1 OS2 | 0.7623 | 0.7632 | 0.756 | 0.7258 | 0.7212 | 0.7155 | 0.6944 | 0.7034 | 0.704 |
| PDL1 OS2 | 0.7274 | 0.7164 | 0.7276 | 0.682 | 0.6789 | 0.6816 | 0.6676 | 0.6641 | 0.6653 |
| PDL1 OS nano1 | 0.7929 | 0.7874 | 0.7506 | 0.7624 | 0.7652 | 0.7552 | 0.7448 | 0.7524 | 0.7433 |
| PDL1 OS nano2 | 0.7418 | 0.7664 | 0.7602 | 0.735 | 0.7293 | 0.7307 | 0.7069 | 0.717 | 0.7129 |
| PDL1 OS nano3 | 0.7423 | 0.7538 | 0.756 | 0.6025 | 0.6088 | 0.604 | 0.5843 | 0.5838 | 0.5841 |
| PDL1 OS 150K1 | 0.803 | 0.8005 | 0.8048 | 0.7786 | 0.7827 | 0.7718 | 0.7586 | 0.7533 | 0.7416 |
| PDL1 OS 150K2 | 0.8068 | 0.8036 | 0.798 | 0.6606 | 0.6558 | 0.6578 | 0.635 | 0.6388 | 0.6458 |

Table 12 Raw data of PDLhTERT calcium deposition ELISA.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PLD1 OS 150K3 | 0.7346 | 0.7419 | 0.7338 | 0.5818 | 0.5812 | 0.5754 | 0.5656 | 0.5648 | 0.5653 |

Table 13 Calcium deposition standard calculation of PDLhTERT.

| | STD + $H_2O$ | Standard concentration (mg/dl) | OD Value (minus blank) |
|---|---|---|---|
| Standard1 | 100 µl + 0 µl | 20 | 0.75103 |
| Standard2 | 80 µl + 20 µl | 16 | 0.61083 |
| Standard3 | 60 µl + 40 µl | 12 | 0.46363 |
| Standard4 | 40 µl + 60 µl | 8 | 0.3186 |
| Standard5 | 30 µl + 70 µl | 6 | 0.2538 |
| Standard6 | 20 µl + 80 µl | 4 | 0.16583 |
| Standard7 | 10 µl + 90 µl | 2 | 0.07446 |
| Blank | 0 µl + 100 µl | 0 | 0 |

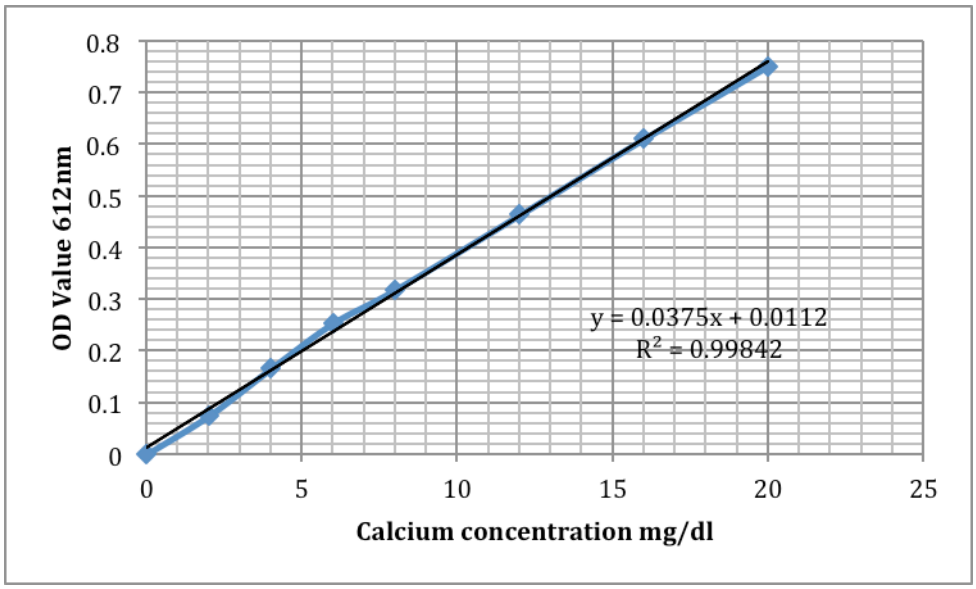


Figure 6 Standard of PDLhTERT Calcium deposition. Standard curve was made according to standards' concentrations and OD values. Samples' calcium concentrations were counted depend on the equation acquired from standard curve.

Standard curve was made according to the known concentration and OD value of the standards (Table 13). Equation was managed from the standard curve (Figure 6). For the standard in this run of PDLhTERT, the equation is:

$$Y=0.0375x + 0.0112$$

$R^2$ shows the accuracy of this equation. $R^2 > 0.95$ is suggested acceptable. So the concentration of the samples would be:

Concentration of sample = (OD of sample – OD of blank – 0.0112)/0.0375

With this equation, all the samples were calculated for their calcium concentration. Samples' calcium concentration were used for future data analysis.

## 2.7 Statistical analysis

SPSS (version 22, SPSS Inc, Chicago, USA), Prism (version 7, Graph Pad Software, San Diego, USA) and Excel (version 14.1.0, Microsoft, Redmond, USA) used for data analysis. Standard deviation (SD) and standard error of mean (SEM) were used to describe the dispersion of the data. Tukey's multiple comparisons test was used to compare the difference between 2 groups. One-way Analysis of variance (ANOVA) was used to analysis among 3 or more group. P values $<0.05$ have been considered significant.

# 3. Results

## 3.1 Flow cytometry

The following figures (Figure 7-15) show the flow cytometry results of cells labeled with CD44, CD90 and CD168 markers.

### 3.1.1 CD44

Both CD44 and CD90 antibodies were with fluorescein. CD90 was used as a surrogate marker for hMSCs and PDLhTERTs. The mean fluorescence intensity (MFI) of CD44 and CD90 were presented as in Figure 7 and 10. Figure 8, 9, 11, 12 showed the patten of cells labeled with CD44 and CD90 in flow cytometry.

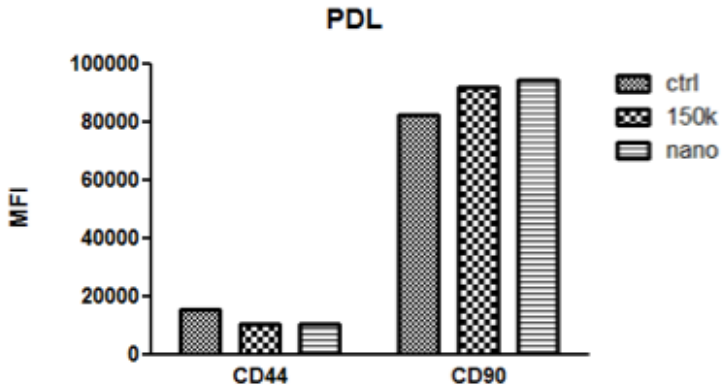


Figure 7 Mean fluorescence intensity (MFI) of PDLhTERTs labeled with CD44 and CD90 markers in flow cytometry. PDLhTERT groups: control, nano HA and 150k HA, stimulated for 7 days.

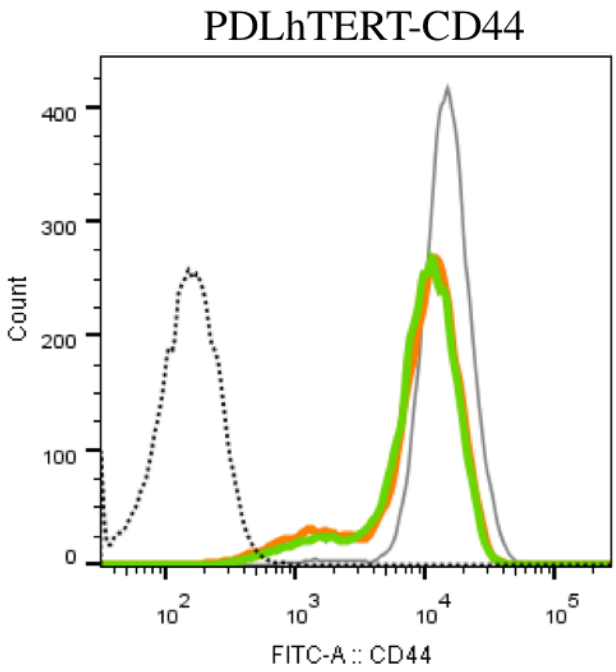


Figure 8 Histogram shows PDLhTRTs labeled with CD44 marker analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

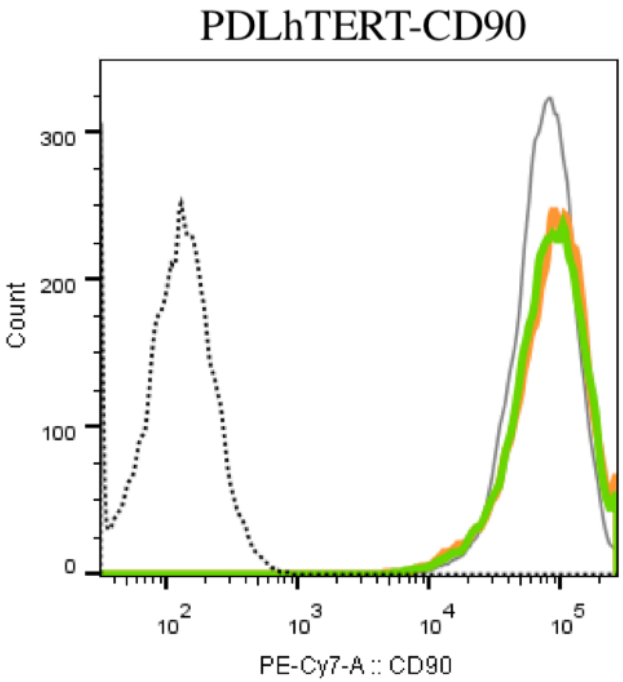

Figure 9 Histogram shows PDLhTRTs labeled with CD90 analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

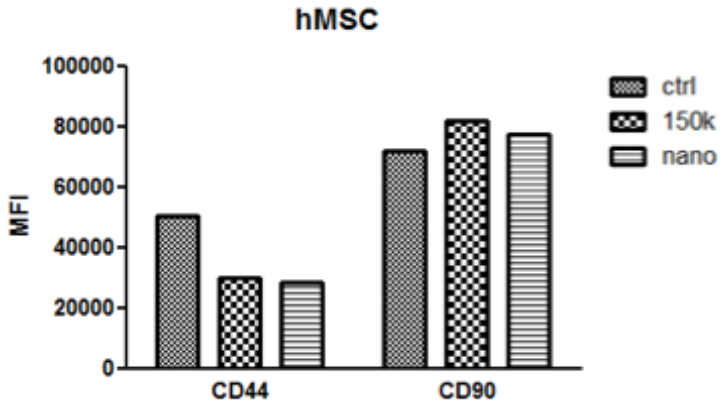


Figure 10 Mean fluorescence intensity (MFI) of hMSCs labeled with CD44 and CD90 markers in flow cytometry. hMSC groups: control, nano HA and 150k HA, stimulated for 7 days. Dotted bar: control; lattice bar: 150k HA; stripes bar: nano HA.

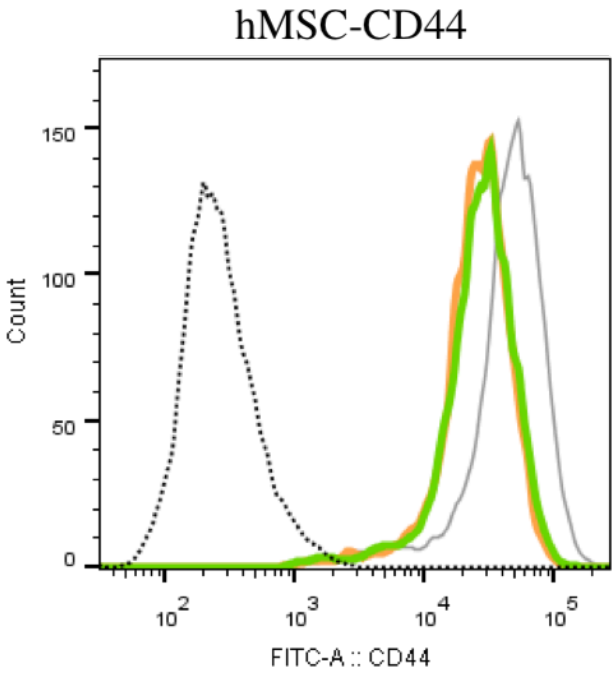


Figure 11 Histogram shows hMSCs labeled with CD44 marker analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

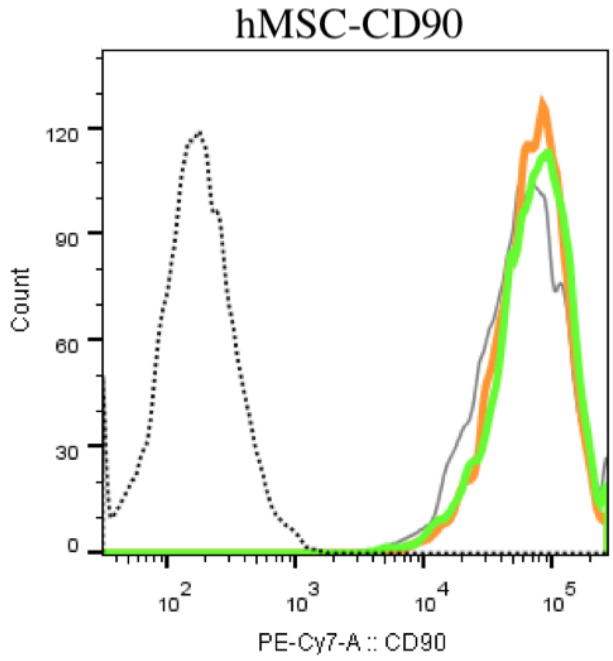


Figure 12 Histogram shows hMSCs labeled with CD90 marker analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

### 3.1.2 CD168

Because CD168 antibody was without fluorescein and incubated with secondary antibody, therefore the percent of positive cells was presented (Figure 13). 150k HA group of PDLhTERTs had the highest positive cells rate while control group of hMSCs was the highest. In Figure 14 and 15 show PDLhTERTs and hMSCs labeled with CD168 analyzed by flow cytometry.

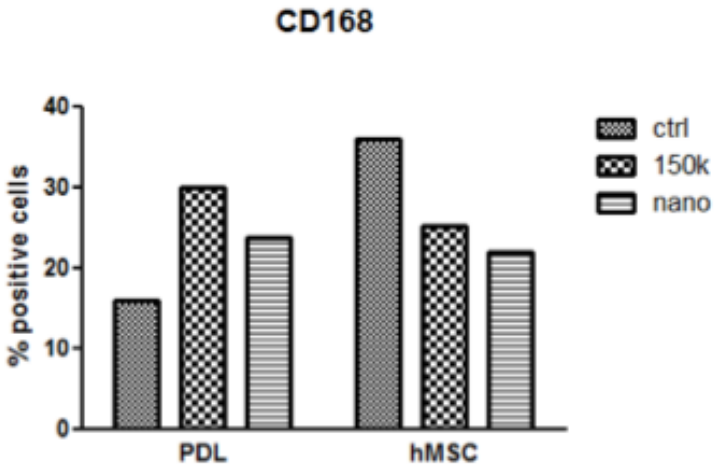


Figure 13 Positive percent cells rate of PDLhTERTs and hMSCs labeled with CD168 marker in flow cytometry. PDLhTERT groups: control, nano HA and 150k HA; hMSC groups: control, nano HA and 150k HA. Both cell types were stimulated for 7 days. Dotted bar: control; lattice bar: 150k HA; stripes bar: nano HA.

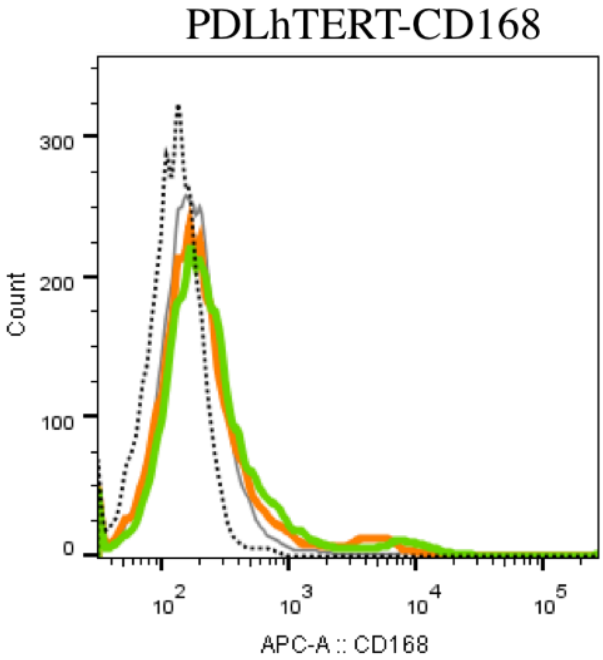


Figure 14 Histogram shows PDLhTRTs labeled with CD168 marker analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

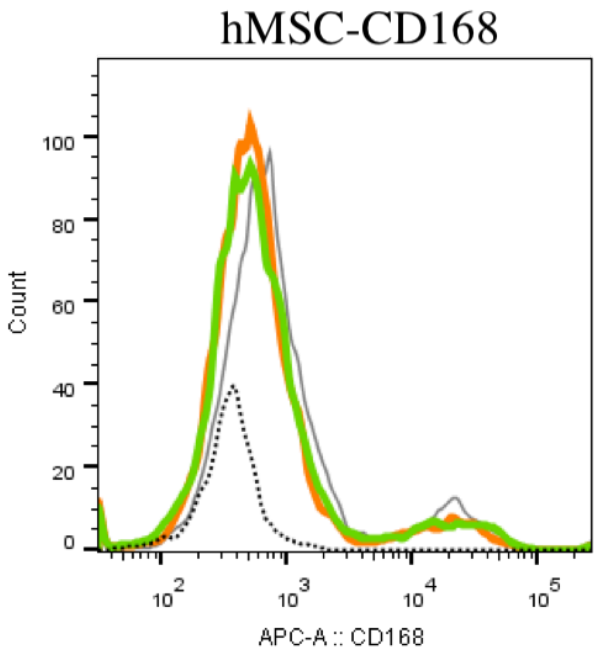


Figure 15 Histogram shows hMSCs labeled with CD168 marker analyzed by flow cytometry. Black dotted line: unstained control; green line: 150k HA; red line: nano HA; grey line: control.

## 3.2 Immunofluorescence analysis

CD44 and CD168 antibodies were analyzed for both cell types. The following figures (Figure 16-19) show the results of immunofluorescence staining.

### 3.2.1 CD44

CD44 is the main HA receptor. hMSCs labeled with CD44 showed prominently positive patten in the whole cytoplasm compared with unlabeled cells, as showed in Figure 16. For PDLhTERTs, the staining was nearly negative in unlabeled groups.

The most prominent staining of PDLhTERT was in nano group (Figure 17).

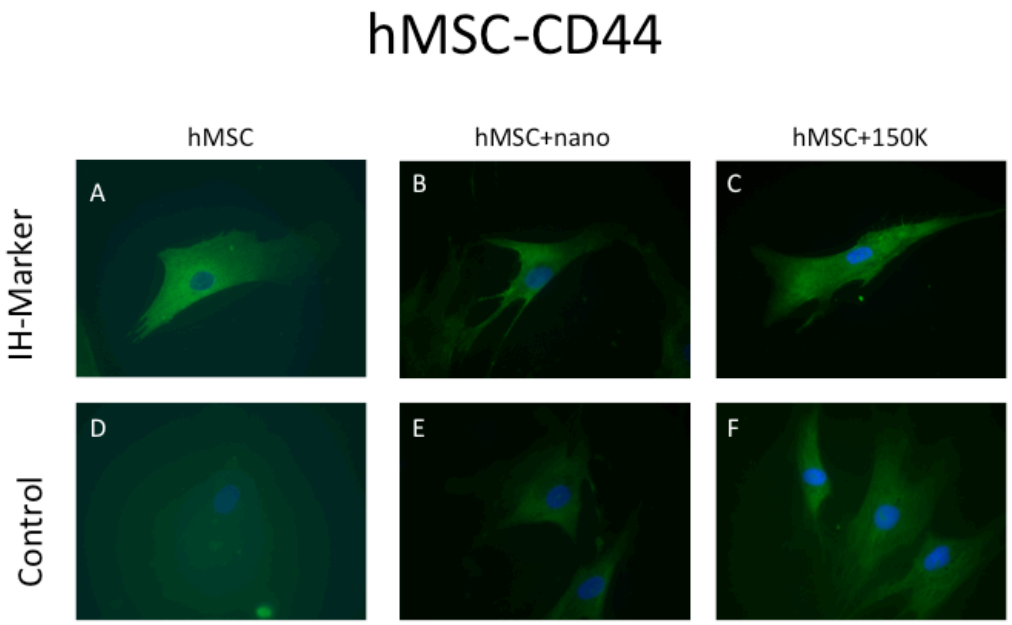


Figure 16 Immunofluorescence analysis of CD44 antibody in hMSC. A: control group labeled with CD44; B: nano HA group labeled with CD44; C: 150k HA group labeled with CD44; D: control without CD44; E: nano HA group without CD44; F: 150k HA group without CD44.

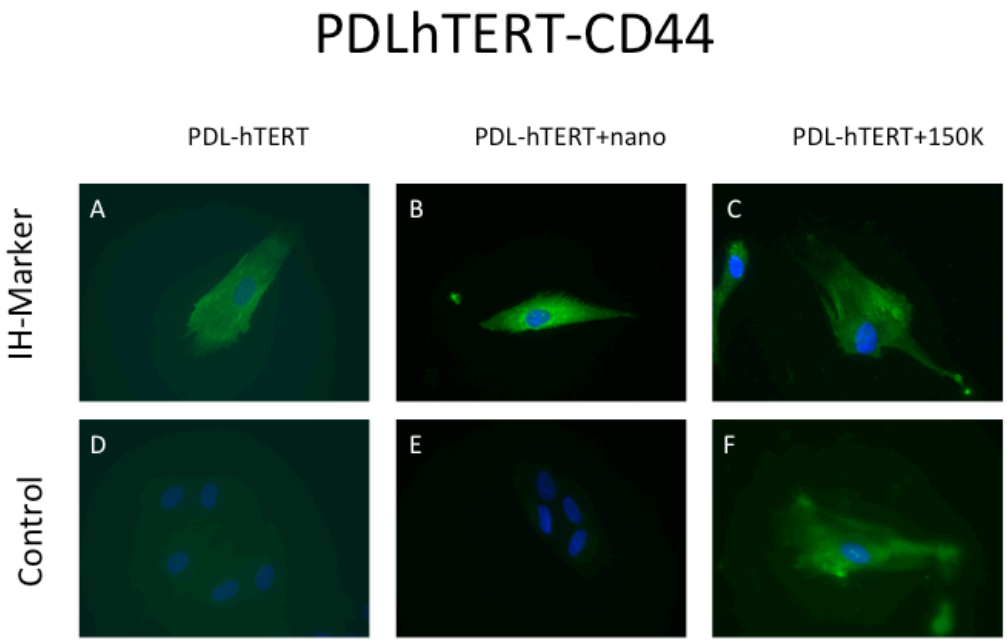


Figure 17 Immunofluorescence analysis of CD44 antibody in PDLhTERT. A: control group labeled with CD44; B: nano HA group labeled with CD44; C: 150k HA group labeled with CD44; D: control without CD44; E: nano HA group without CD44; F: 150k HA group without CD44.

### 3.2.2 CD168

CD168 is also a very important HA receptor. hMSCs labeled with CD44 showed prominently positive patten in the whole cytoplasm compared with unlabeled cells (Figure 18). For PDLhTERTs, the staining was nearly negative in unlabeled groups. The most prominent staining of PDLhTERT was in nano group (Figure 19).

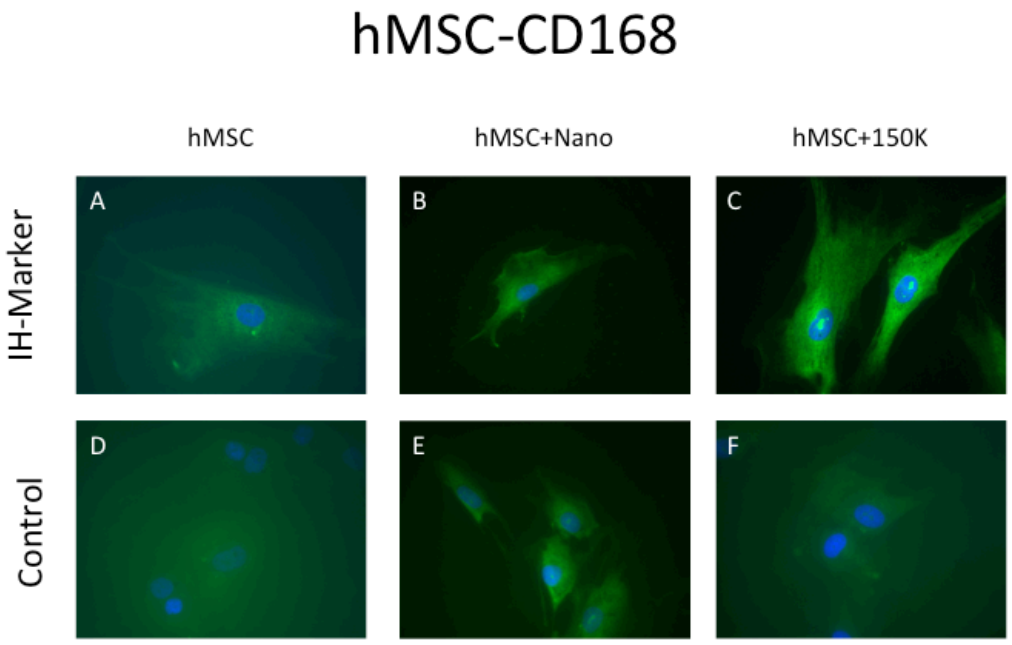


Figure 18 Immunofluorescence analysis of CD168 antibody in hMSC. A: control group labeled with CD44; B: nano HA group labeled with CD44; C: 150k HA group labeled with CD44; D: control without CD44; E: nano HA group without CD44; F: 150k HA group without CD44.

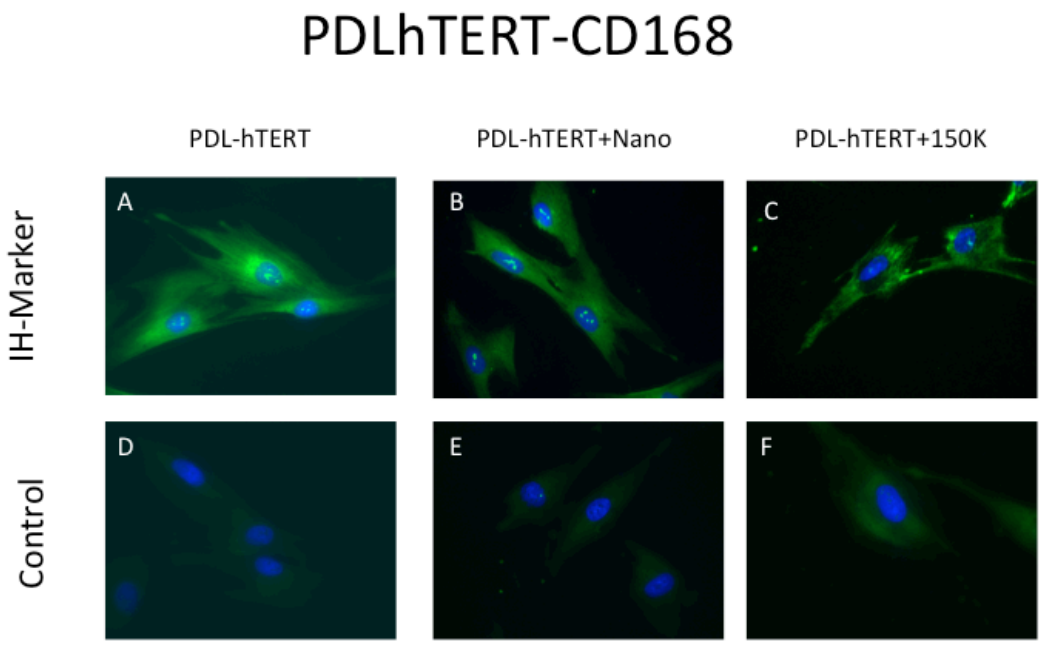


Figure 19 Immunofluorescence analysis of CD168 antibody in PDLhTERT. A: control group labeled with CD44; B: nano HA group labeled with CD44; C: 150k HA group labeled with CD44; D: control without CD44; E: nano HA group without CD44; F: 150k HA group without CD44.

## 3.3 Von Kossa stain

After 21 days , both cells formed tight membranes attached to the bottom of the wells. In von Kossa stain, different shade of color means different mineral deposition. As showed in Figure 20, there was barely any deposition of mineral aggregate in both control groups of two cell types. Therefore controls were more bright than other working groups.

Among hMSC groups, +OS group showed deepest color followed by +OS+150k HA and +OS+nano HA group. As for PDLhTERTs groups, +OS+150k HA group seem to have the highest mineral aggregates, followed by +OS and +OS+nano HA groups. Von Kossa stain is an observational measurement of the mineral aggregates, quantitative measurement can be seen in the following calcium deposition analysis.

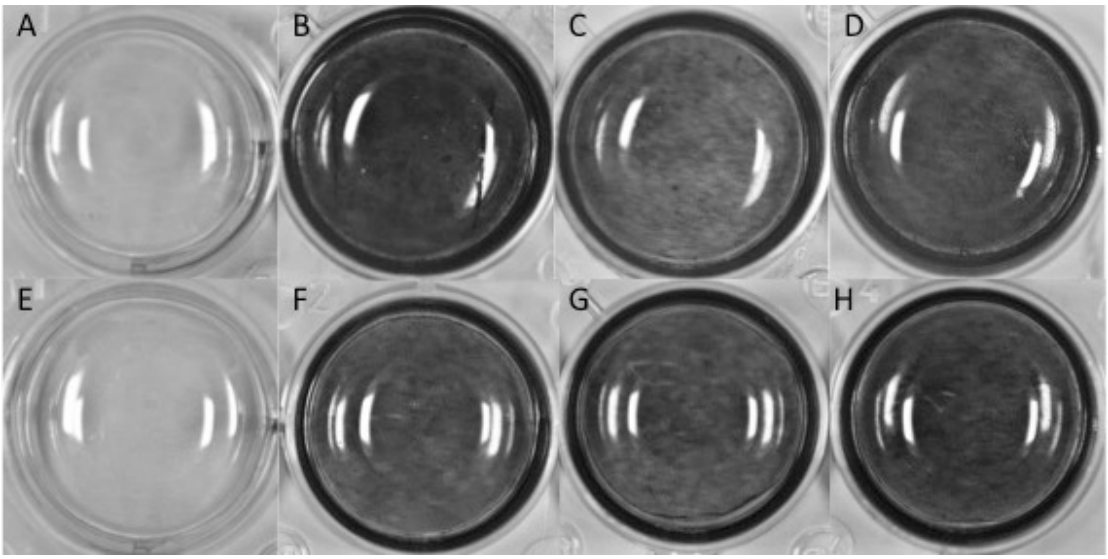


Figure 20 Von Kossa stain for hMSCs and PDLhTERTs after 21 days of cell culture. The deeper the color, the more mineral aggregates deposition. A: hMSC Control group; B: hMSC +OS group; C: hMSC +OS+nano HA HA group; D: hMSC +OS+150k HA group. E: PDLhTERT Control group; F: PDLhTERT +OS group; G: PDLhTERT +OS+ nano HA group; H: PDLhTERT +OS+150k HA group. Both control groups showed barely any mineral aggregates deposition. Among hMSCs groups: +OS **>** +OS+150kDa HA **>** +OS+nano HA . PDLhTERTs groups: +OS+150kDa HA **>** +OS **>** +OS+nano HA.

## 3.4 Calcium deposition analysis

Calcium deposition analysis is a quantitative measurement of calcium concentration of the cells. All the data were calculated as mentioned in materials and the final results of hMSCs are presented in Figure 21. For hMSC groups, the highest calcium concentration was obtained in the +OS group while the +OS+nano HA group was the lowest. Both +OS+nano HA and +OS+150kDa HA groups showed decreased expression of calcium compared with OS group (both $P < 0.0001$). +OS+nano HA expressed lower than +OS+150kDa HA groups ($P < 0.0001$). All the groups had significantly higher calcium deposition than control group ($P < 0.0001$).

For PDLhTERT groups, as shown in Figure 22, the highest calcium expression seemed to be +OS+150kDa HA but there was no statistical significant difference when compared with OS group ($p = 0.2426$). However +OS+nano HA decreased the expression of calcium and was significant when compared to +OS group ($p < 0.0001$). +OS+150kDa HA also had higher expression than +OS+nano HA group ($p < 0.0001$). All the groups had significantly higher calcium deposition than control group ( $p<0.0001$).

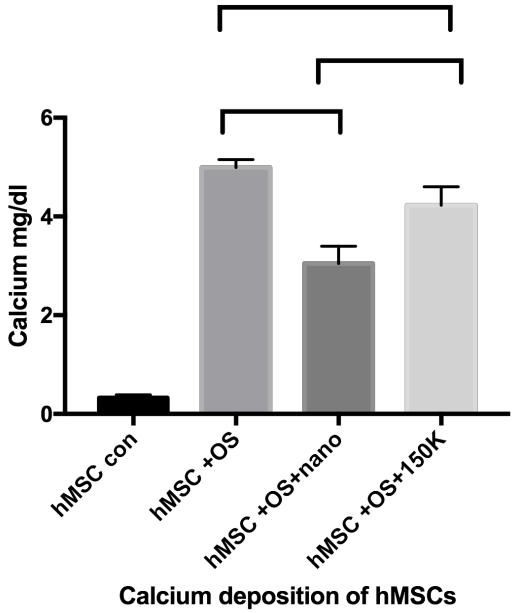


Figure 21 Calcium deposition results of hMSCs. Columns are representing the mean ± standard error (SD) of calcium deposits. The working groups had higher calcium expression than control groups ($p<0.0001$). Two groups with a comparison marker overhead had statistically significant difference ($p<0.0001$).

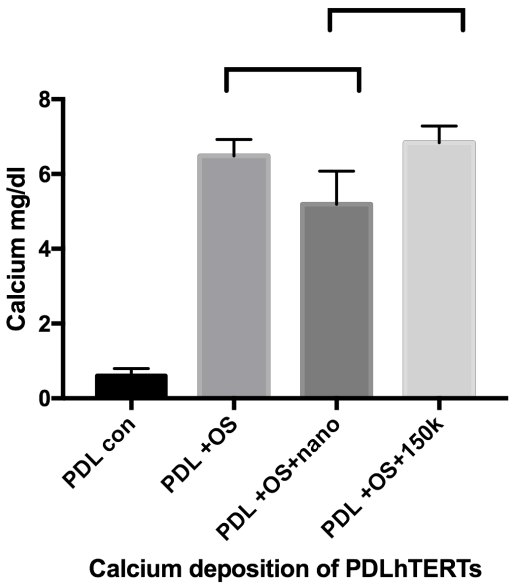


Figure 22 Calcium deposition results of PDLhTERTs. Columns are representing the mean ± standard error (SD) of calcium deposits. The working groups had higher calcium expression than control groups ($p<0.0001$). Two groups with a comparison marker overhead had statistically significant difference ($p<0.0001$).

# 3.5 PCR results

## 3.5.1 CD44

In hMSCs the CD44 expression remained roughly unchanged during the entire observation period independent on the specific stimulation conditions, as in Figure 23. At day 21, the control group also expressed significantly less CD44 than +OS and +OS+150kDa HA groups ($p=0.0002$, $p=0.0012$), however no difference was found between +OS and HA groups. At day 3 and 21, the comparisons among all four groups were significant ($p=0.0335$; $p<0.0001$).

Obviously PDLhTERTs' CD44 expressions all peaked at day 7, as presented in Figure 24. CD44 expression was attenuated by stimulation. At day 3, +OS+nano and +OS+150k HA groups were significantly less expressed than +OS group (p=0.0117, p=0.0046). At day 7 nano HA was less expressed than +OS group (1p=0.0149); 150kDa HA was also less than control, but no difference with +OS group. The comparisons among all four groups at day 3, 7 and 21 were all significant (p<0.0001; p=0.0009; p<0.0001).

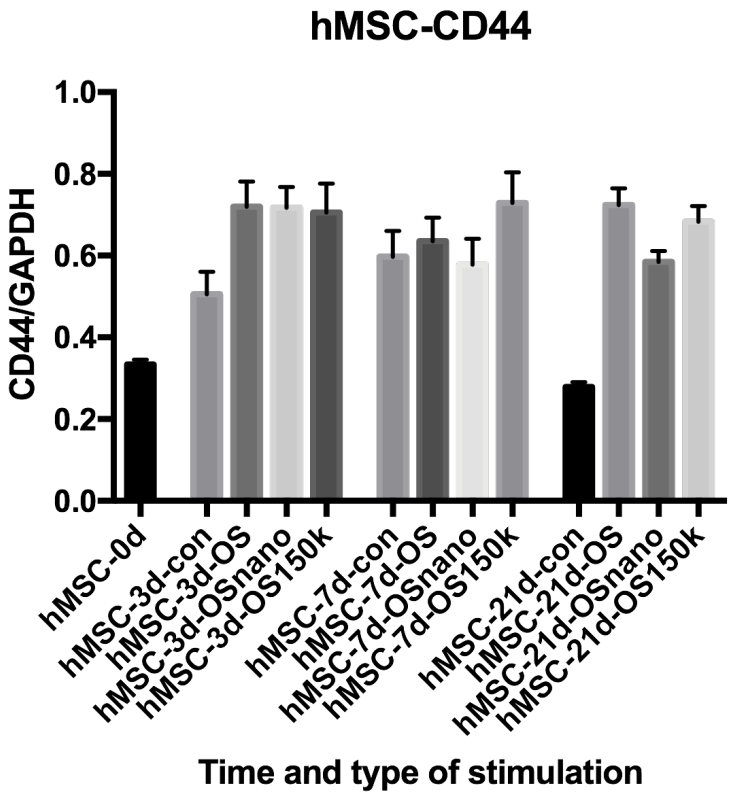

Figure 23 Columns relative CD44 expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. In hMSCs the CD44 expression remained roughly unchanged during the entire observation period independent on the specific stimulation conditions.

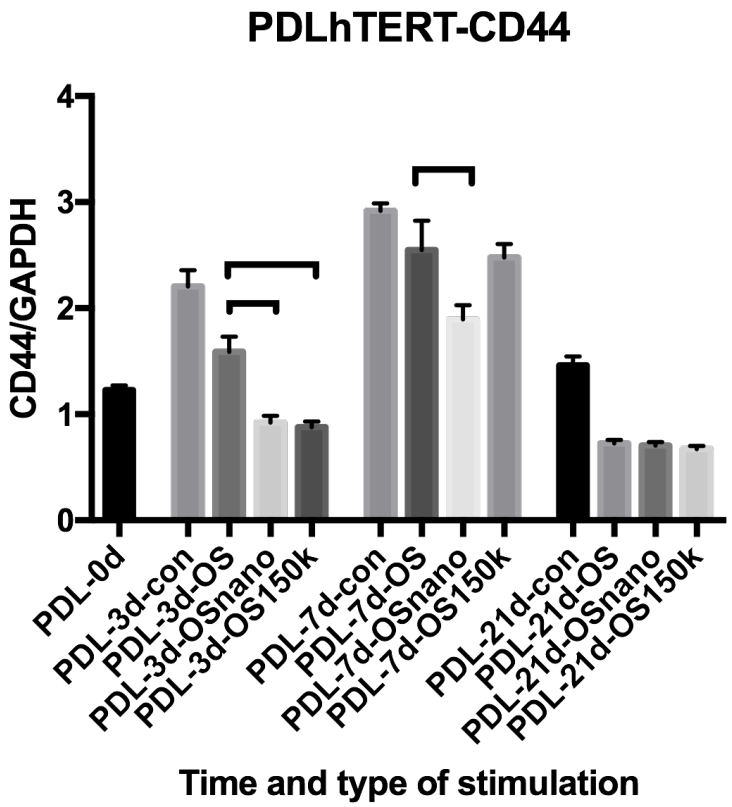


Figure 24 Columns relative CD44 expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 3, +OS+nano and +OS+150k HA groups were significantly less expressed than OS group ($p=0.0117$, $p=0.0046$). At day 7 nano HA was less expressed than OS group ($p=0.0149$); 150kDa HA was also less than control, but no difference with OS group.

### 3.5.2 CD168

CD168 expression trends of hMSCs and PDLhTERTs were similar. For both the +OS group was the highest at day 3, especially the +OS group of PDLhTERTs.

For hMSCs (Figure 25), CD168 expression was unaffected by stimulation. The comparisons among all four groups at day 7 and 21 were all significant ($p=0.0251$; $p<0.0001$).

For PDLhTERTs CD168 expression was inhibited by HA (Figure 26). At day 3, compared with +OS group, +OS+nano HA and +OS+150k HA depressed CD168 expression (both $p<0.0001$), however no difference was found between +OS+nano HA and +OS+150k HA groups. The comparisons among all four groups at day 3 and 7 were all significant ($p<0.0001$; $p=0.0009$).

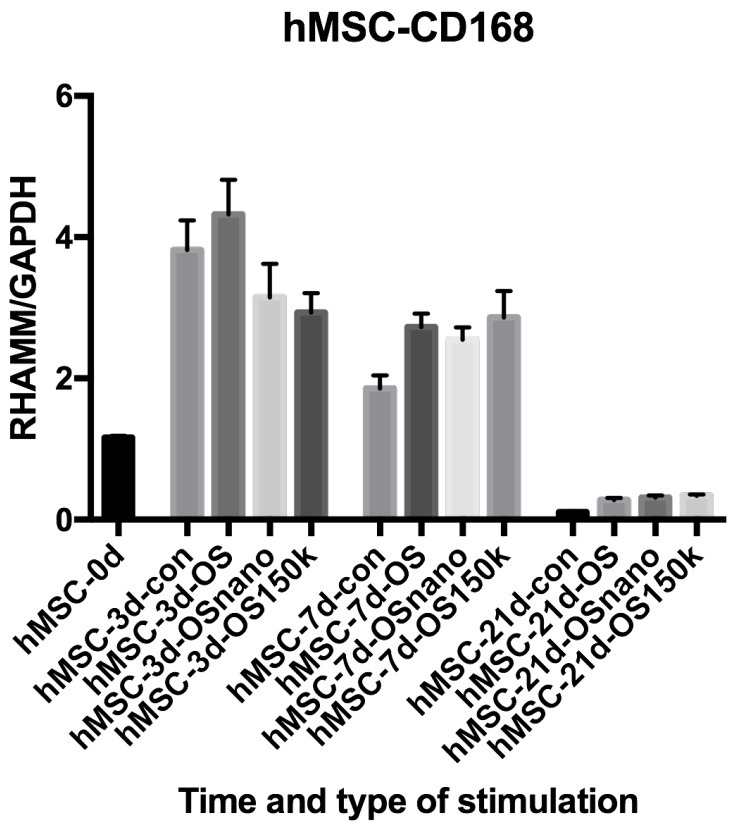


Figure 25 Columns relative CD168 expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. hMSCs' CD168 expression was almost unaffected by stimulation.

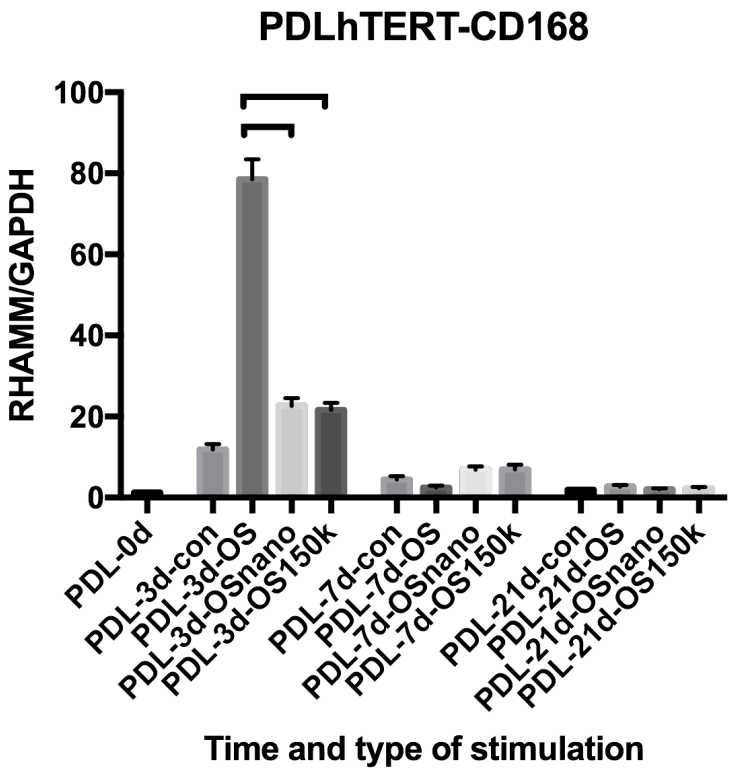


Figure 26 Columns relative CD168 expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. HA inhibited PDLhTERTs' CD168 expression. Compared with +OS group, +OS+nano HA and +OS+150k HA depressed CD168 expression (both $p<0.0001$) at day 3.

### 3.5.3 TLR4

hMSCs and PDLhTERTs showed almost the same trends of TLR4 expression. HA almost had no effect at first 3 time points and attenuated TLR4 expression at day 21.

For hMSCs (Figure 27), at day 21, +OS+nano HA group was significantly lower expressed than +OS group ($p<0.0001$). The comparisons among all four groups at day 3, 7 and 21 were all significant ($p=0.0003$; $p=0.0244$; $p<0.0001$).

In PDLhTERTs (Figure 28), HA seemed to have no effect during early stage stimulation, however attenuated TLR4 expression at day 21. At day 21, +OS+nano HA and +OS+150k HA group significantly attenuated TLR4 expression compared with +OS group ($p<0.0001$, $p=0.0004$). No significant difference was found between +OS+nano HA and +OS+150k HA group. The comparisons among all four groups at day 3, 7 and 21 were all significant (all $p<0.0001$).

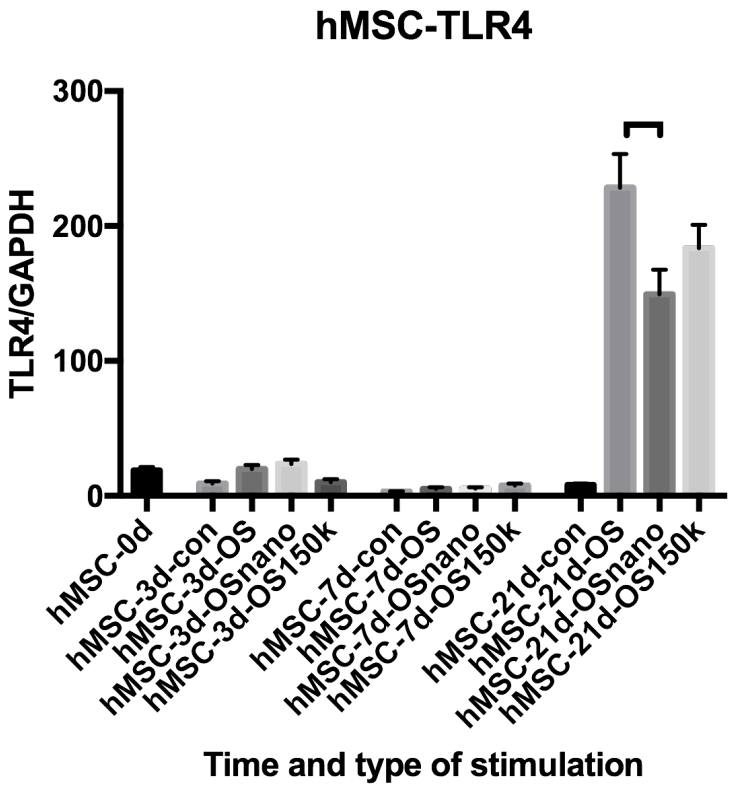


Figure 27 Columns relative TLR4 expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 21, +OS+nano HA group was significantly lower expressed than +OS group ($p<0.0001$).

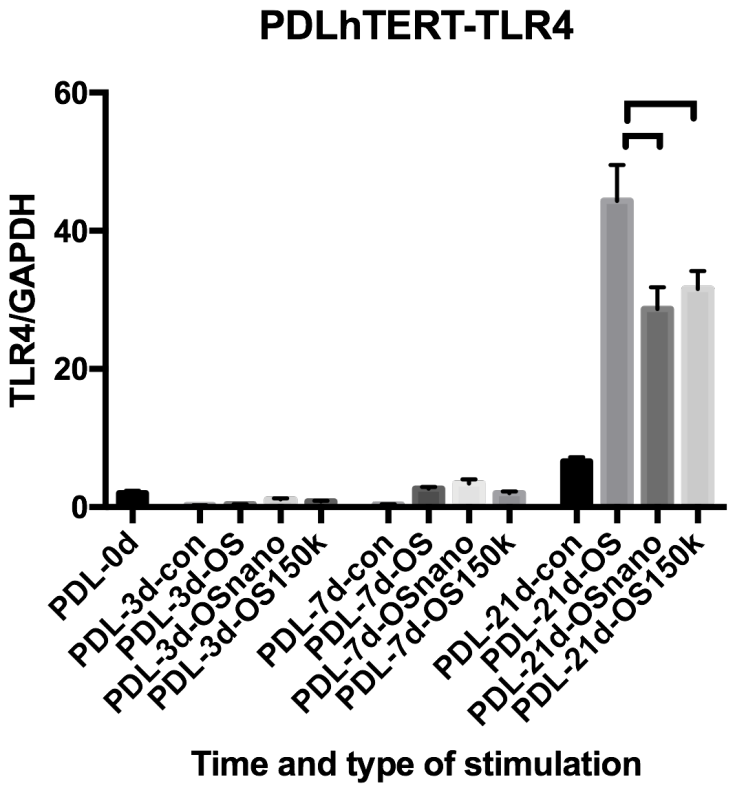


Figure 28 Columns relative TLR4 expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. In PDLhTERTs HA seemed to have no effect during early stage stimulation, however attenuated TLR4 expression at day 21. At day 21, +OS+nano HA and +OS+150k HA group significantly attenuated TLR4 expression compared with +OS group ($p<0.0001$, $p=0.0004$).

### 3.5.4 CAP

For hMSCs, (Figure 29) HA groups seemed to have no effect on CAP expression. At day 7, 150kDa HA had significantly higher expression than control ($p<0.0001$). At

day 21, all the working groups, +OS, +OS+nano HA and +OS+150k HA, expressed more CAP than control group ($p<0.0001$, $p=0.0035$, $p=0.0004$), however no significant difference among working groups. The comparisons among all four groups at day 3, 7 and 21 were all significant ($p<0.0001$, $p=0.0002$, $p<0.0001$).

For PDLhTERTs (Figure 30), CAP expression was inhibited by HA. At day 7, +OS and +OS+150k HA groups had higher expression than +OS+nano HA group ($p=0.0002$, $p=0.0084$), however no difference between +OS and +OS+150k HA groups. The comparisons among all four groups at day 3, 7 and 21 were all significant ($p<0.0001$, $p= 0.014$, $p<0.0001$).

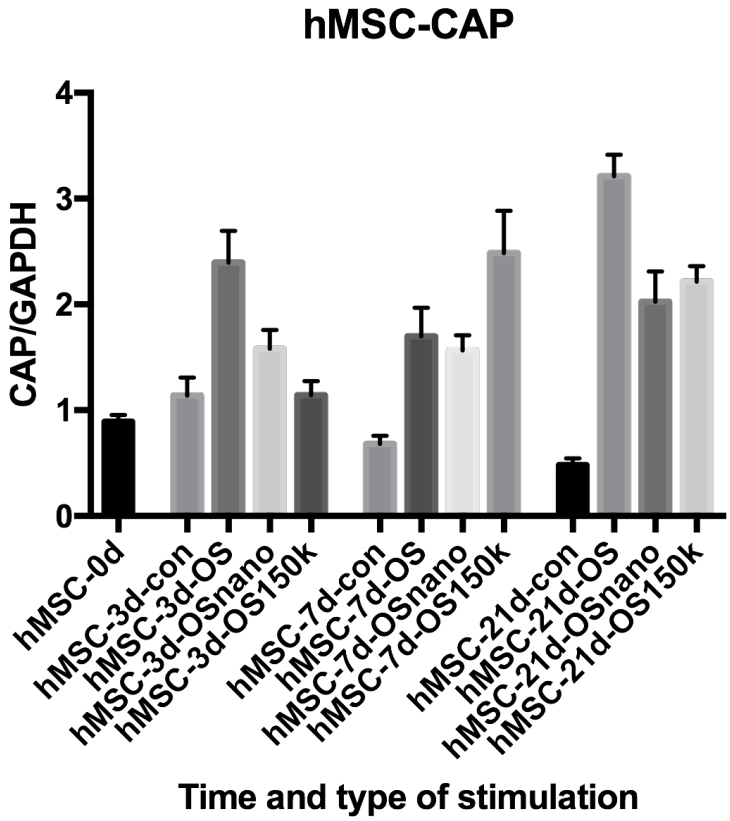

Figure 29 Columns relative CAP expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. For hMSCs, stimulation seemed to have no effect on CAP expression.

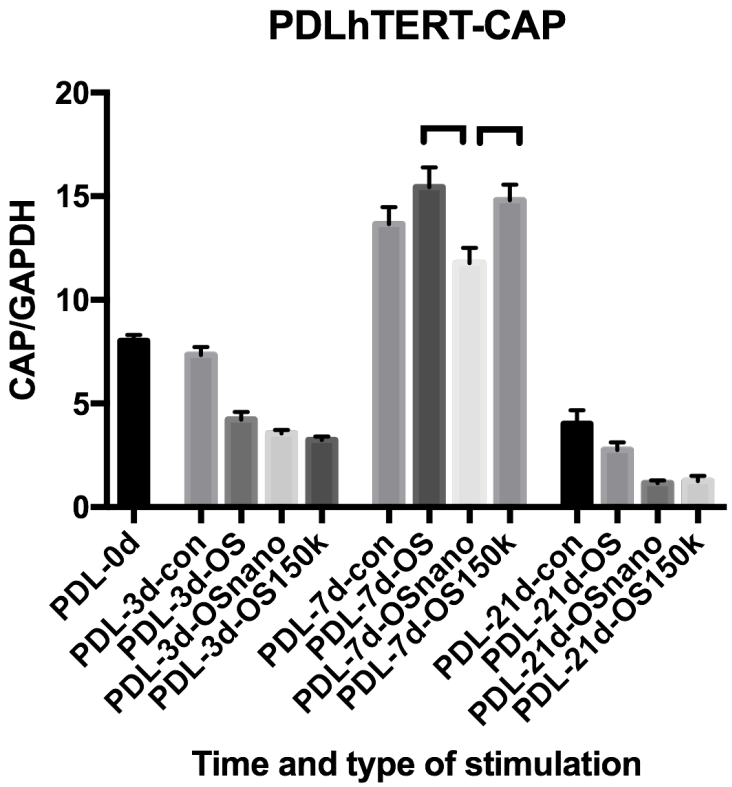


Figure 30 Columns relative CAP expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 7, +OS and +OS+150k HA groups had higher expression than +OS+nano HA group ($p=0.0002$, $p=0.0084$).

### 3.5.5 CEMP1

Both nano and 150kDa HA inhibited hMSCs' CEMP1 expression (Figure 31). At day 7 CEMP1 expressions of +OS and +OS+150k HA groups were prominently higher than +OS+nano HA group ($p<0.0001$, $p=0.0069$). At day 21, +OS group expression was prominently higher than +OS+nano HA and +OS+150k HA group ($p<0.0001$, $p=0.0046$), however no difference was found between +OS+150k HA and +OS+nano HA groups. The comparisons among all four groups at day 7 and 21 were significant (both $p<0.0001$).

For PDLhTERTs the peak of CEMP1 expression was at day 7 (Figure 32). At day 7, +OS group was significantly higher than +OS+nano HA and +OS+150k HA groups ($p<0.0001$, $p=0.0181$), however no difference was found between nano and 150kDa HA. The comparisons among all four groups at day 3, 7 and 21 were all significant ($p=0.0002$, $p=0.0003$, $p<0.0001$).

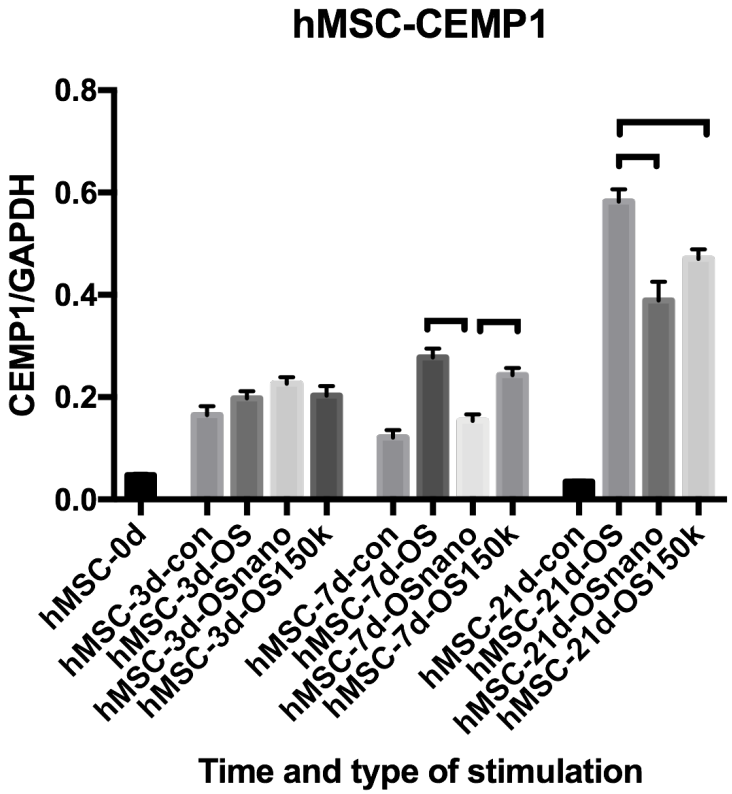


Figure 31 Columns relative CEMP1 expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. Both nano and 150kDa HA inhibited hMSCs' CEMP1 expression. At day 7 CEMP1 expressions of +OS and +OS+150k HA groups were prominently higher than +OS+nano HA group ($p<0.0001$, $p=0.0069$). At day 21, +OS group CEMP1 expression was prominently higher than +OS+nano HA and +OS+150k HA group ($p<0.0001$, $p=0.0046$).

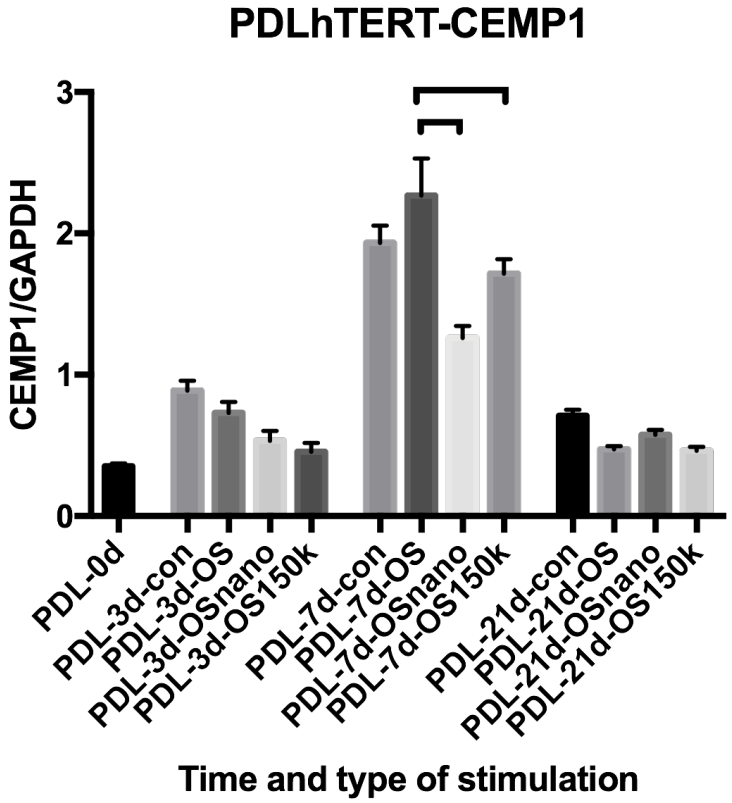


Figure 32 Columns relative CEMP1 expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 7, +OS group was significantly higher than +OS+nano HA and +OS+150k HA groups ($p<0.0001$, $p=0.0181$).

### 3.5.6 SCX

Both hMSCs and PDLhTERTs had very low expression of SCX. In the current study the expression of SCX remained almost unchanged irrespective of the specific

stimulation condition for both cells.

For hMSCs (Figure 33), stimulation almost had no effect on its SCX expression. At d ay 3 control was also significantly higher than +OS+nano HA group (p= 0.0229). At day 21, +OS group expressed higher SCX than control group (p=0.0445). No significant difference was found among working groups. The comparisons among all four groups at day 3 and 21 were significant (p=0.0087, p<0.0001).

PDLhTERTs expressed more SCX than hMSCs (Figure 34). However no difference was found between any groups during all 21 days of culture. Only the comparisons among all four groups on 21 was significant (p=0.0021).

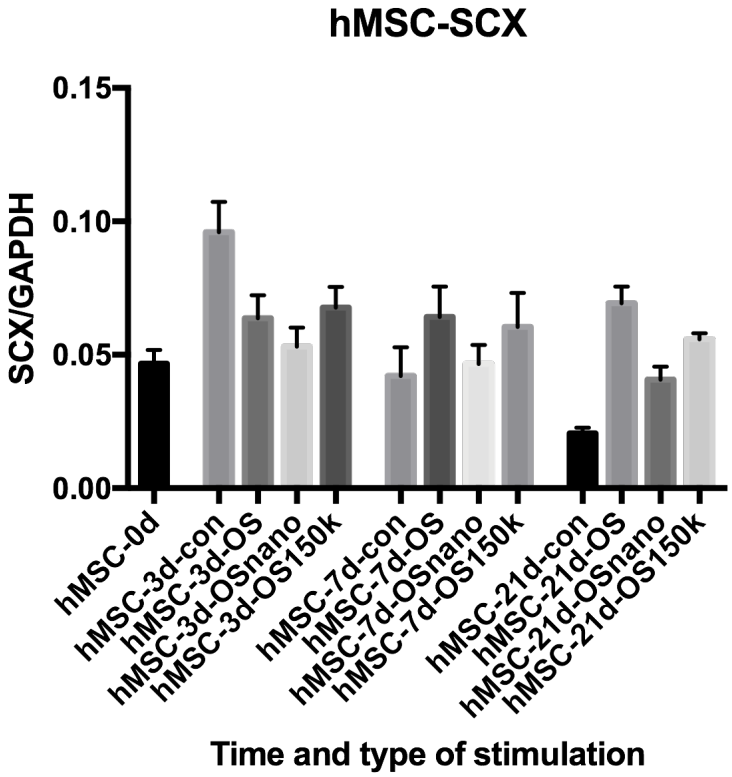


Figure 33 Columns relative SCX expression [mean ± standard error of mean (SEM)] of

hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. HA stimulation almost had no effect on hMSCs' SCX expression.

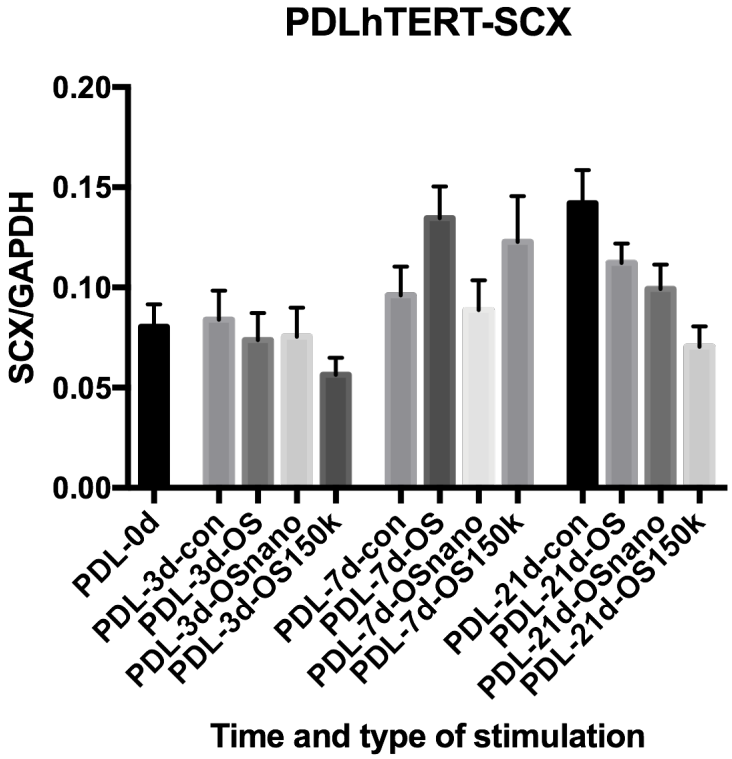


Figure 34 Columns relative SCX expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. PDLhTERTs expressed SCX, however no difference was found between any groups during all 21 days of culture.

### 3.5.7 ALP

hMSCs and PDLhTERTs showed completely opposite trends of ALP expression.

For hMSCs (Figure 35), at day 3 and 7 +OS+nano HA expressed more ALP than the +OS group of the same time points ($p=0.0348$, $p=0.0254$). At day 21, both +OS+nano HA and +OS+150k HA had higher expression than +OS group ($p=0.0332$, $p<0.0001$); +OS+150k HA was also statistically higher than +OS+nano HA ($p=0.0063$). Control groups of each time points were significantly lower expressed than working groups. The comparisons among all four groups at day 3, 7 and 21 were all significant (all $p< 0.0001$).

PDLhTERTs had very low and flat ALP expression on first 3 time points, but increased markedly at day 21, especially +OS+nano HA and +OS+150k HA groups (Figure 36). At day 21, both +OS+nano HA and +OS+150k HA expressed more ALP than +OS group (both $p<0.0001$), but no significant expression between +OS+nano HA and +OS+150k HA. The comparisons among all four groups at day 3, 7 and 21 were all significant ($p=0.0299$, $p<0.0001$, $p< 0.0001$).

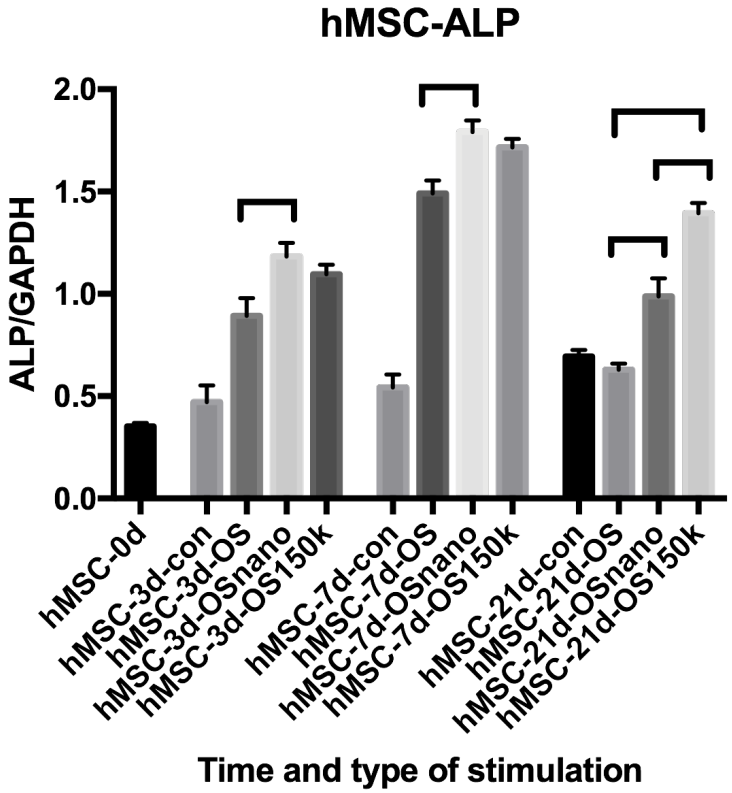


Figure 35 Columns relative ALP expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 3 and 7 hMSCs +OS+nano HA expressed more ALP than the +OS group of the same time points ($p=0.0348$, $p=0.0254$). At day 21, both +OS+nano HA and +OS+150k HA had higher expression than +OS group ($p=0.0332$, $p<0.0001$); +OS+150k HA was also statistically higher than +OS+nano HA ($p=0.0063$).

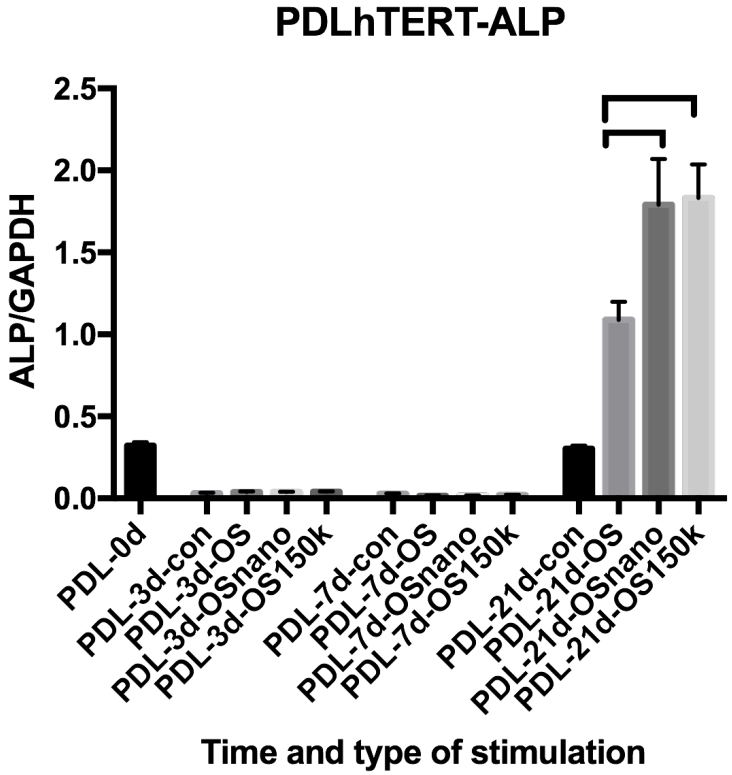


Figure 36 Columns relative ALP expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 21, both +OS+nano HA and +OS+150k HA expressed more ALP than +OS group (both $p<0.0001$).

### 3.5.8 BSP

HA seemed to have no effect on hMSCs' BSP expression (Figure 37). At day 7, hMSCs' +OS+150k group expressed more BSP than +OS+nano HA ($p<0.0001$), however no difference was found between +OS+150k HA and +OS groups. There

was no significant difference of BSP expression among groups, the only exception was day 7 (p=0.0039).

For PDLhTERTs (Figure 38), stimulation also had no effect on BSP expression. There was no significant difference of BSP expression among groups from day 0 to day 7, the only exception was day 21 (p=0.0002).

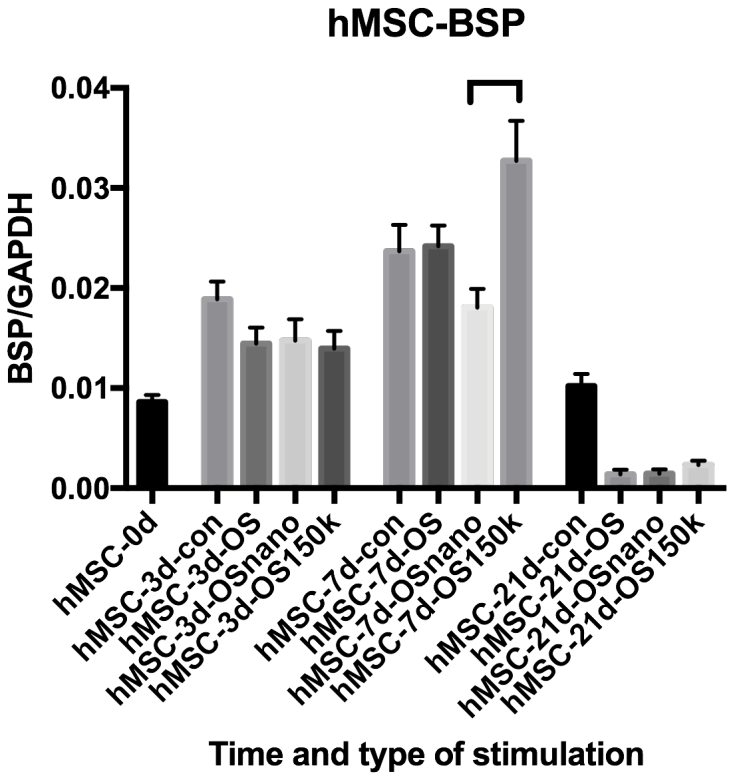


Figure 37 Columns relative BSP expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. HA seemed to have no effect on hMSCs' BSP expression. At day 7, +OS+150k HA expressed

more BSP than +OS+nano HA ($p<0.0001$).

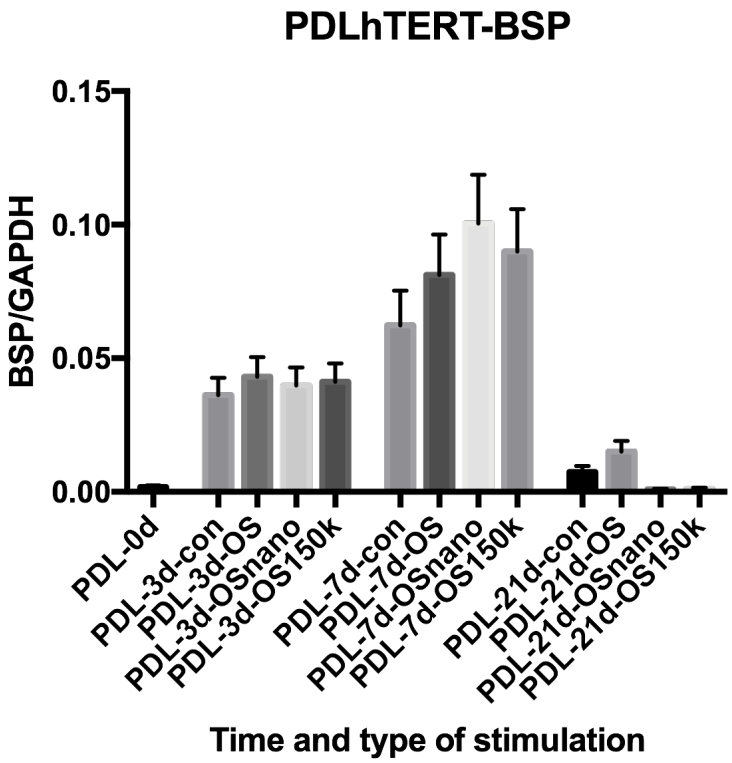


Figure 38 Columns relative BSP expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. Standard deviation was used for all data. HA seemed to have no effect on PDLhTERTs' BSP expression.

### 3.5.9 COL1A1

hMSCs and PDLhTERTs showed opposite COL1A1 expression trends during 21 days of cell culture.

For hMSCs (Figure 39), +OS+nano group had higher COL1A1 expression than +OS group at day 3 ($p=0.001$). The comparisons among all four groups at day 3 and 7 were significant ($p=0.0095$, $p<0.0001$).

For PDLhTERTs (Figure 40), at day 21, +OS+150k HA group expressed the highest amount of COL1A1. +OS+150k HA had significantly higher COL1A1 expression than +OS+nano and +OS group (np=0.0111, $p<0.0001$). +OS+nano HA was also higher than +OS group ($p=0.0056$). The comparisons among all four groups at day 3, 7 and 21 were all significant ($p=0.0007$, $p<0.0001$, $p=0.0016$).

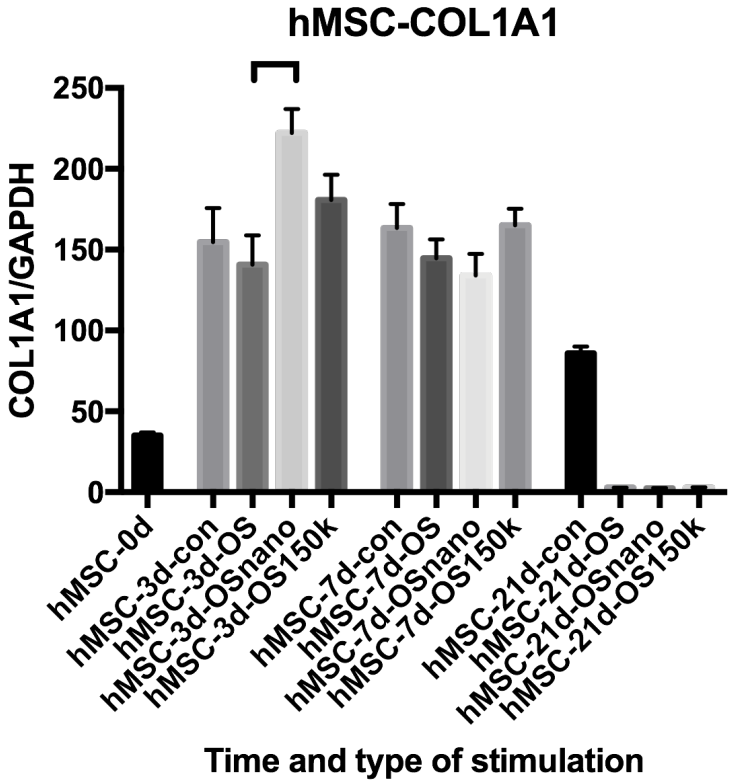


Figure 39 Columns relative COL1A1 expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. +OS+nano group had higher COL1A1 expression than +OS group at day 3 ($p=0.001$).

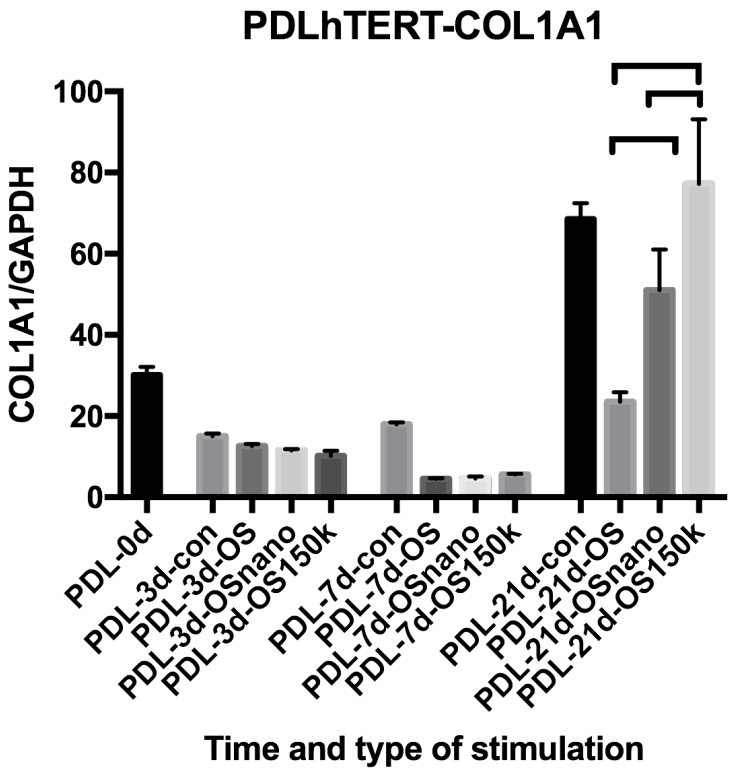


Figure 40 Columns relative COL1A1 expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 21, +OS+150k HA group expressed the highest amount of COL1A1. +OS+150k HA had significantly higher COL1A1 expression than +OS+nano and +OS group ($p=0.0111$, $p<0.0001$). +OS+nano HA was also higher than +OS group ($p=0.0056$).

### 3.5.10 OCN

On both, day 3 and 7, +OS+nano HA group of hMSC expressed higher OCN than +OS group ($p=0.002$, $p=0.0031$), as showed in Figure 41. However +OS+150k HA had no significant difference when compared with +OS group. The comparisons

among all four groups at day 3, 7 and 21 were all significant (p=0.0051, p=0.0088, p<0.0001).

The OCN expression of PDLhTERTs was attenuated by 150kDa HA stimulation (Figure 42). At day 7, +OS group was higher expressed than +OS+150k HA group (p=0.026). The comparisons among all four groups at day 7 and 21 were significant (p<0.0001, p=0.0009).

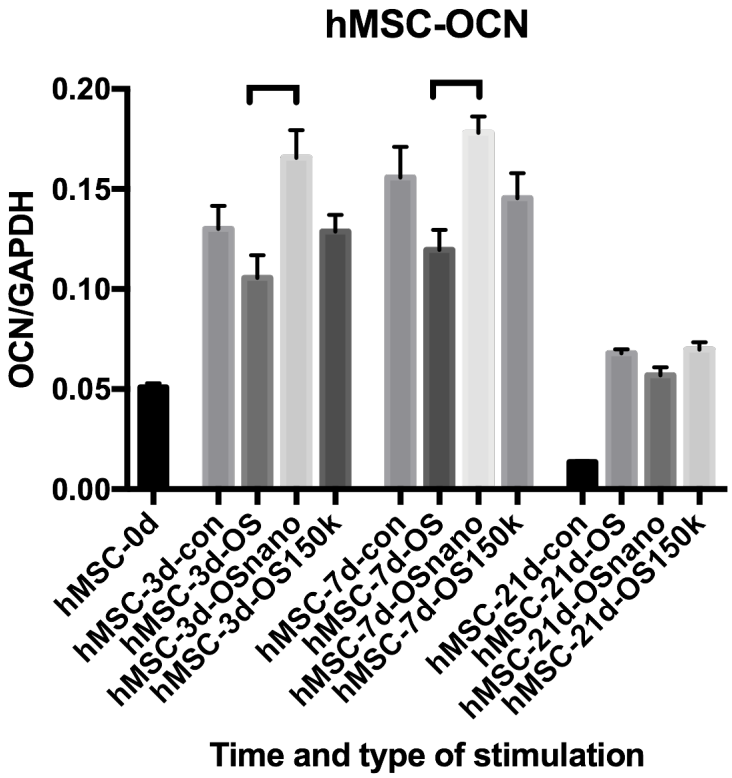


Figure 41 Columns relative OCN expression [mean ± standard error of mean (SEM)] of hMSCs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. On

both day 3 and 7, +OS+nano HA expressed higher OCN than +OS group (p=0.002, p=0.0031).

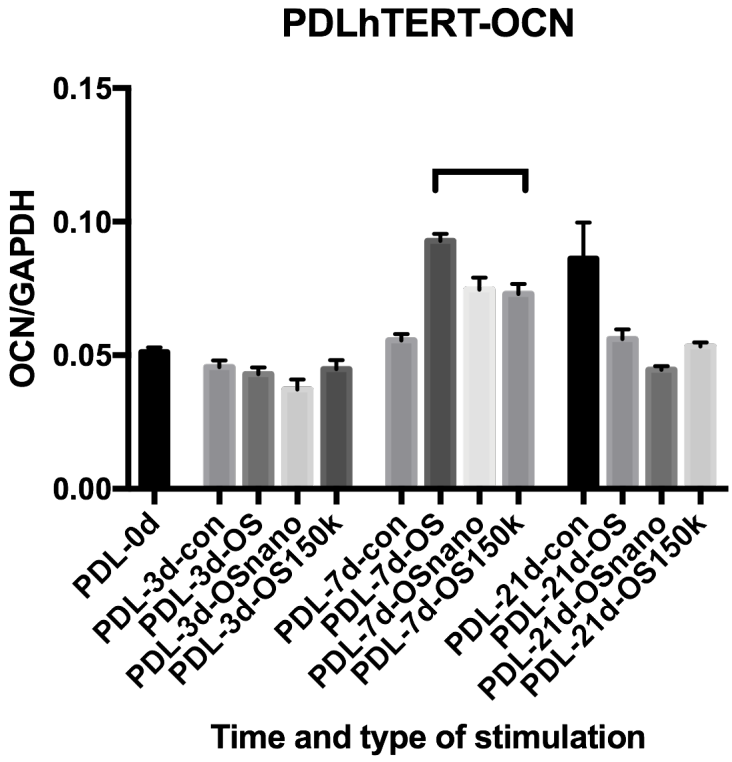


Figure 42 Columns relative OCN expression [mean ± standard error of mean (SEM)] of PDLhTERTs for control, +OS, +OS+nano and +OS+150k groups which cultured for 0, 3, 7 and 21 days. Relative CD 44 expression was normalized against GAPDH as housekeeping gene. At day 7, +OS group was higher expressed than +OS+150k HA group (p=0.026).

# 4. Discussion

This study aimed to delineate the effects of low molecular weight fragments of HA on the cementogenic, ligamentogenic and osteogenic differentiation of PDLhTERTs and hMSCs. Both types of cells can be found within periodontal tissues and were, accordingly, suggested to play a central role in the regeneration of periodontal defects (Silverio et al. 2008, Suaid et al. 2011, Sanchez-Lara 2013). For periodontal tissue regeneration, stem cells have to differentiate into osteoblasts, periodontal ligament cells, and cementoblasts (Maeda et al. 2011). Comparable as in wounds also in pathogenic tissue defects native high molecular weight HA is fragmented during tissue repair (McAtee et al. 2014, Parsons 2015). In comparison to native HA the low molecular fragments have the potential to interact with stem cells and to promote their in-trafficking into the healing tissue defect (Kota et al. 2014, Veiseh et al. 2015).

## 4.1 HA markers

Among other marker molecules, which are characteristic for mesenchymal stem cells, specifically the CD44 receptor is highly expressed in these cells (Choi et al. 2015). CD44 is the main cell surface receptor interacting with HA (Aruffo et al. 1990) which, upon binding to HA, regulates various biological functions including proliferation and differentiation (Viola et al. 2015). CD168/CD138 represents the second major receptor for HA (Cheung et al. 1999). Due to alternative splicing also this receptor is expressed in various isoforms depending on the specific cell type

(Kavasi et al. 2017). Unlike the CD44 receptor the CD168 receptor is only sparsely expressed on the cell surface (Nikitovic et al. 2016). In-vitro experiments on human keratinocytes revealed that HA enhances the expression of both, the CD44 and the HMMR gene showing a strong positive correlation with the molecular weight of HA (D'Agostino et al. 2017). In fibrocytes HMW-HA increased the expression of CD44 receptors but opposed to that LMW-HA caused a significant inhibition (Maharjan et al. 2011). Herein, both receptor genes have been substantially expressed in both cell types. In hMSCs the expression, however, remained roughly unchanged during the entire observation period independent on the specific stimulation conditions. On the contrary in PDLhTERTs the presence of small oligosaccharides nano and/or the 150kDa HA fragment significantly attenuated the expression of the CD44 receptor, however no difference was found between nano and 150kDa HA. A recent study has reported that the proliferation and mineralization capacities of PDL cells are inevitably bound to the presence of the CD44 receptor (Yeh et al. 2014). Based on these results it can be assumed that the differentiation of PDLhTERTs is more advanced than that of hMSCs consequently being more susceptible to stimuli modifying the expression of CD44. Despite being classified as pluripotent stem cells, both types of cells have a different ontogenetic nature (Proksch et al. 2012, Proksch et al. 2014). Whereas hMSCs can be considered as true mesenchymal cells, PDLhTERTs are derived from the neural crest and are therefore assigned to have an ectomesenchymal origin. Hence, in comparison to hMSCs the PDLhTERTs might have a less undifferentiated phenotype reacting more instantly on extrinsic stimuli

inducing cell differentiation.

Consistent results have been obtained for the expression of the CD168 receptor, which has been almost unaffected in hMSCs but was inhibited by HA in PDLhTERTs. OS medium enhanced PDLhTERTs' CD168 expression. Both nano and 150kDa HA inhibited CD168 expressions, however no significant difference was found between these two different molecular weight HA. Recent observations on mesenchymal progenitor cells revealed that the expression of the CD44 and the CD168 receptor are closely interrelated (Veiseh et al. 2015). Specifically the CD168 receptor seems to control the CD44 expression along with the perception of HA. Hence, linkage between the expression of CD44 and the CD168 receptor might explain the comparable changes under HA stimulation in PDLhTERTs.

TLRs were reported to be able to activate keratinocytes' reaction toward injury without CD44, the main receptor (Gariboldi et al. 2008). The up regulation of TLR4 may activate NF-κB in the mouse MSCs, which will increase prostaglandin E2 (PGE2) secretion and finally enhance inflammation (Prockop et al. 2012). A former study indicated that TLR4 expression of hMSCs were almost the same after 1 and 7 days of osteogenic stimulation, but decreased slightly after 14 days (Ebert et al. 2015). In this study TLR4 expressions of both hMSCs and PDLhTERTs were attenuated by nano and/or 150k HA. OS medium enhanced TLR4 expression for both cells. TLR4 blocking was also reported to inhibit osteogenetic differentiation of MSCs when cultured with OS medium (Herzmann et al. 2017).

## 4.2 Cementogenic differentiation

For the initiation of periodontal regeneration including the re-formation of fibrous attachment the development of new cementum on the root surface comprises the central step (Saygin et al. 2000). Yet, two marker molecules, i.e. CAP and CEMP1 have been identified to be specifically expressed in cementogenic cells (Liu et al. 1997, Alvarez-Perez et al. 2006). Osteogenic stimulation was reported to inhibit CAP and CEMP1 expression of PDL stem cells at day 15. Extra supplement of vitamin C (VC) can reverse inhibition and enhance cementogenic differentiation (Gauthier et al. 2017).

In this study, HA inhibited cementogenic differentiation of both cell types. HA had no effect on hMSCs' CAP expression; while nano HA obviously inhibited PDLhTERTs' CAP expression. Nano and 150kDa HA attenuated CEMP1 expression of both hMSCs and PDLhTERTs. PDLhTERTs showed the highest expression of both cementogenic marker molecules already after day 7 whereas in hMSCs an increasing expression was found until day 21. This observation, again, can be explained by biological differences between both types of stem cells leading to a varying responsiveness to extrinsic growth stimuli (Luan et al. 2009). Despite these potential phenotypic differences the presence of HA reduced the transcription of the cementogenic marker molecules in both cell types.

## 4.3 Ligamentogenic differentiation

Scleraxis is a transcription factor that can be found in progenitor cells of tendon and in PDL cells. Typically it is used as marker molecule indicating differentiation of stem cells towards periodontal ligament fibroblasts, i.e. (Seo et al. 2004, Inoue et al. 2012). In the current study the expression of this marker remained almost unchanged irrespective of the specific stimulation condition for both hMSCs and PDLhTERTs.

## 4.4 Osteogenic differentiation

OS medium can enhance ALP, BSP, COL1A1 and OCN expressions in hMSCs (Sila-Asna et al. 2007). All the experimental groups were with OS medium in this study. Regarding the osteogenic differentiation the expression of ALP was induced by the various stimulation conditions in both, hMSCs and PDLhTERTs. Intriguingly, it was considerably stronger in the presence of oligosaccharide nano and 150 kDa HA. HA accelerated hMSCs' ALP expression obviously, 150kDa HA was even stronger than nano HA. In contrast to the cementogenic marker molecules the expression of ALP was significantly stronger in hMSCs already at shorter periods of time as compared to PDLhTERTs. PDLhTERTs' ALP expression was increased markedly by HA at day 21, however no difference was found between nano and 150kDa HA.The specific origin of hMSCs from the bone marrow might provide a plausible explanation for their prompter osteogenic differentiation. ALP is considered as marker molecule indicating the early mineralization process by hydrolyzing phosphate esters

subsequently accumulating phosphate ions within the ECM (Malaval et al. 1999, Viereck et al. 2002).

BSP contributes to bone, cementum and dentin mineralization and has angiogenic capacity (Ogata 2008, Bouleftour et al. 2016). BSP can be used in collagen scaffold coating to increase bone tissue repair ( Kruger et al. 2013). Therefore BSP expression can be very important for periodontal regeneration. However in this study HA seemed to have no effect on both cells' BSP expression.

Except for BSP also the expression of the osteogenic marker molecules representing later stages of osteogenic differentiation, i.e. COL1A1 and OCN seemed to be enhanced by the HA fragments.

Type I collagen is an essential component of the dermis, bone, and tendon matrix. COL1A1 was also considered as an early marker of osteogenic differentiation (Weinreb et al. 1990). HMW-HA was reported to express less COLL1A1 than LMW-HA and high-low molecular HA mix complex at day 2 (D'Agostino et al. 2015). In this study, nano HA seemed to be able to increase hMSCs' COL1A1 expression at day 3. For PDLhTERTs, COL1A1 expression was increased by both nano and 150kDa HA at day 21, especially 150kDa HA. A study of hBMSCs cultured with HA hydrogels showed increased COL1A1 and OCN expressions with longer time of stimulation and higher HA concentration in the hydrogels (Jung et al. 2018). Administration route, HA molecule weight and HA concentration may effect HA's function on cells.

OCN was the only late osteogenic differentiation marker of the four chosen

markers in this work and has been described to be essential for bone formation (Ducy et al. 1996). In this study the OCN expression of hMSCs was increased by nano HA, however 150kDa HA seemed to have no effect on it. For PDLhTERTs, 150kDa HA slightly attenuated OCN expression.

In general, HA roughly increased ALP, COL1A1 and OCN expressions of hMSCs and PDLhTERTs. For OCN, nano HA increased its expression in hMSCs but 150kDa HA decreased it in PDLhTERTs. Nano HA seemed always to be an accelerator while 150kDa HA sometimes was an inhibitor. As we know hMSCs have more osteogenic differentiation potential than PDLhTERTs (Docheva et al. 2010, Egusa et al. 2012). The difference of HA effected osteogenic markers expressions may be because hMSCs were earlier differentiated (in first 7 days) than PDLhTERTs (started from day 21). Stem cell differentiation direction and degree were effected by many factors in the environment, such as cell morphology, cell density, virus infection, stimulant, and differentiation media. Cell density will effect cell shape and influence differentiation directly (McBeath et al. 2004). In this study cells were seeded in the same density and without virus infection. However hMSCs and PDLhTERTs growth velocity were different, which may lead to different densities at later stimulation. This may also explain the difference of expressions between hMSC and PDLhTERT.

Calcium deposition is commonly suggested as end-stage osteogenic marker. Regarding this marker both, the osteogenic stimulation alone and together with HA lead to the highest calcium deposition. In both cell types there was, however, a trend for an inhibitory effect on calcium deposition for HA which seemed to be stronger for

the nano HA as compared to the 150 kDa HA. Partially in line with the current results, in osteoblastic cells of rodents the 60 kDa HA did not cause changes in ALP activity but induced a significant stronger transcription of osteocalcin as compared to unstimulated controls (Huang et al. 2003). In addition, the HA with the lower molecular mass of 60 kDa did not enhance the mineralization as compared to the high molecular mass HA of 900 kDa and 2300 kDa respectively. Contradictory to the present results another study using porcine bone marrow derived mesenchymal cells observed a strong reduction of the expression of osteogenic differentiation markers, i.e. COLIA1 and ALP, in the presence of HA. However, in this study the HA used for stimulation and leading to a significant enhancement of calcium deposition after 21 days had a considerably higher molecular mass of 900 kDa (Zou et al. 2008).

# 5. Conclusion

Periodontitis is a highly prevalent chronic inflammatory oral disease which leads to bone loss, attachment loss and, ultimately, to tooth loss. HA is a non-sulfated glycosaminoglycan that can be found extensively in human tissue including periodontal tissue. HA was reported to have significant influence on periodontal tissue repair and has already been widely used in medical applications. This study aimed to delineate the effects of various sized HA molecules on periodontal cells hMSCs and PDL-hTERTs. The HA receptors CD44, CD168 and the TLR4 have been substantially expressed in both cell types. In hMSCs CD44 and CD168 expression remained roughly unchanged during the entire observation period; in PDLhTERTs small nano and/or the 150 kDa HA fragment significantly attenuated the expression of the CD44 receptor. TLR4 expression was inhibited by nano and/or 150kDa HA in both cell types at day 21. In addition, the presence of HA reduced the transcription of the cementogenic marker molecules in both cell types, especially nano HA. SCX, a ligamentogenic marker, remained almost unchanged irrespective of the specific stimulation condition. Early stage osteogenic marker ALP was induced by the various stimulation conditions in both hMSCs and PDLhTERTs and stronger in the presence of nano and 150 kDa HA. BSP remained roughly unchanged under stimulation. Osteogenic markers COL1A1 in both cell types and OCN in hMSCs were also enhanced by the HA fragments. However OCN expression in PDLhTERTs it was inhibited by 150k HA. The osteogenic stimulation alone and together with HA lead to the highest calcium deposition.

Taken together the current study revealed that small HA fragments cause differential effects on hMSCs and PDLhTERTs. Nano HA seemed to have more positive effects in osteogenic differentiation than 150kDa HA. These fragments seem to enhance the earlier steps of osteogenic differentiation in both types of cells but to impair the expression of cementogenic differentiation markers and the mineralization of the ECM during osteogenesis within 21 days. Since the expression of SCX was unaffected HA seems to have no influence on the ligamentogenesis.

# 6. Supplementary data

Here were rt-qPCR results of 10 primers, Table 14 included HA receptors CD44, CD168 and TLR4; Table 15 included OS markers ALP, BSP, COL1A1 and OCN; Table 16 included cementogenic and ligamentogenic markers CAP, CEMP1 and SCX. Comparison between two samples was used Tukey's multiple comparisons test. Comparison of four groups at the same time point was used One-way ANOVA, presented in Table 17. PDLhTERT was simply written as 'PDL' in all following tables.

Table 14 PCR positive results of HA receptors

| Tukey's multiple comparisons test | | Mean Diff. | 95.00% CI of diff. | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| CD44 | hMSC-7d-con vs. hMSC-21d-con | 0.3189 | 0.0386 to 0.5992 | * | 0.0113 |
| | hMSC-21d-con vs. hMSC-21d-OS | -0.4449 | -0.752 to -0.1379 | *** | 0.0002 |
| | hMSC-21d-con vs. hMSC-21d-OS150k | -0.4043 | -0.7113 to -0.09726 | ** | 0.0012 |
| | PDL-3d-con vs. PDL-3d-OS | 0.6173 | 0.02333 to 1.211 | * | 0.0335 |
| | PDL-3d-con vs. PDL-3d-OSnano | 1.282 | 0.6876 to 1.876 | **** | <0.0001 |

Table 14 PCR positive results of HA receptors

| | | | | |
|---|---|---|---|---|
| PDL-3d-con vs. PDL-3d-OS150k | 1.327 | 0.7328 to 1.921 | **** | <0.0001 |
| PDL-3d-con vs. PDL-7d-con | -0.7148 | -1.309 to -0.1209 | ** | 0.0051 |
| PDL-3d-con vs. PDL-21d-con | 0.7425 | 0.06286 to 1.422 | * | 0.0189 |
| PDL-3d-OS vs. PDL-3d-OSnano | 0.6642 | 0.07878 to 1.25 | * | 0.0117 |
| PDL-3d-OS vs. PDL-3d-OS150k | 0.7095 | 0.1241 to 1.295 | ** | 0.0046 |
| PDL-3d-OS vs. PDL-7d-OS | -0.9605 | -1.546 to -0.3751 | **** | <0.0001 |
| PDL-3d-OS vs. PDL-21d-OS | 0.8631 | 0.2086 to 1.518 | ** | 0.0011 |
| PDL-3d-OSnano vs. PDL-7d-OSnano | -0.9728 | -1.558 to -0.3874 | **** | <0.0001 |
| PDL-3d-OS150k vs. PDL-7d-OS150k | -1.602 | -2.187 to -1.016 | **** | <0.0001 |
| PDL-7d-con vs. PDL-7d-OSnano | 1.024 | 0.4381 to 1.609 | **** | <0.0001 |
| PDL-7d-con vs. PDL-21d-con | 1.457 | 0.7852 to 2.129 | **** | <0.0001 |
| PDL-7d-OS vs. PDL-7d-OSnano | 0.6519 | 0.06648 to 1.237 | * | 0.0149 |
| PDL-7d-OS vs. PDL-21d-OS | 1.824 | 1.169 to 2.478 | **** | <0.0001 |
| PDL-7d-OSnano vs. PDL-21d-OSnano | 1.19 | 0.5354 to 1.844 | **** | <0.0001 |
| PDL-7d-OS150k vs. PDL-21d-OS150k | 1.806 | 1.151 to 2.46 | **** | <0.0001 |
| PDL-21d-con vs. PDL-21d-OS | 0.738 | 0.004854 to 1.471 | * | 0.0468 |
| PDL-21d-con vs. PDL-21d-OSnano | 0.7562 | 0.02305 to 1.489 | * | 0.0363 |

Table 14 PCR positive results of HA receptors

| | | | | | |
|---|---|---|---|---|---|
| | PDL-21d-con vs. PDL-21d-OS150k | 0.7887 | 0.05554 to 1.522 | * | 0.0227 |
| CD168 | hMSC-3d-con vs. hMSC-7d-con | 1.96 | 0.5664 to 3.354 | *** | 0.0003 |
| | hMSC-3d-con vs. hMSC-21d-con | 3.713 | 2.154 to 5.271 | **** | <0.0001 |
| | hMSC-3d-OS vs. hMSC-7d-OS | 1.593 | 0.1989 to 2.987 | * | 0.0106 |
| | hMSC-3d-OS vs. hMSC-21d-OS | 4.043 | 2.485 to 5.601 | **** | <0.0001 |
| | hMSC-3d-OSnano vs. hMSC-21d-OSnano | 2.838 | 1.28 to 4.396 | **** | <0.0001 |
| | hMSC-3d-OS150k vs. hMSC-21d-OS150k | 2.592 | 1.034 to 4.151 | **** | <0.0001 |
| | hMSC-7d-con vs. hMSC-21d-con | 1.752 | 0.194 to 3.311 | * | 0.0131 |
| | hMSC-7d-OS vs. hMSC-21d-OS | 2.45 | 0.892 to 4.009 | **** | <0.0001 |
| | hMSC-7d-OSnano vs. hMSC-21d-OSnano | 2.235 | 0.677 to 3.794 | *** | 0.0002 |
| | hMSC-7d-OS150k vs. hMSC-21d-OS150k | 2.525 | 0.9671 to 4.084 | **** | <0.0001 |
| | PDL-3d-con vs. PDL-3d-OS | -66.68 | -74.97 to -58.38 | **** | <0.0001 |
| | PDL-3d-con vs. PDL-3d-OSnano | -10.73 | -18.9 to -2.556 | ** | 0.0012 |
| | PDL-3d-con vs. PDL-3d-OS150k | -9.807 | -17.98 to -1.633 | ** | 0.0053 |
| | PDL-3d-con vs. PDL-21d-con | 9.925 | 0.7867 to 19.06 | * | 0.0202 |
| | PDL-3d-OS vs. PDL-3d-OSnano | 55.95 | 47.65 to 64.24 | **** | <0.0001 |
| | PDL-3d-OS vs. PDL-3d-OS150k | 56.87 | 48.58 to 65.16 | **** | <0.0001 |

Table 14 PCR positive results of HA receptors

| | | | | | |
|---|---|---|---|---|---|
| | PDL-3d-OS vs. PDL-7d-OS | 76.12 | 67.71 to 84.53 | **** | <0.0001 |
| | PDL-3d-OS vs. PDL-21d-OS | 75.84 | 66.59 to 85.08 | **** | <0.0001 |
| | PDL-3d-OSnano vs. PDL-7d-OSnano | 15.72 | 7.546 to 23.89 | **** | <0.0001 |
| | PDL-3d-OSnano vs. PDL-21d-OSnano | 20.53 | 11.39 to 29.67 | **** | <0.0001 |
| | PDL-3d-OS150k vs. PDL-7d-OS150k | 14.68 | 6.508 to 22.86 | **** | <0.0001 |
| | PDL-3d-OS150k vs. PDL-21d-OS150k | 19.43 | 10.29 to 28.57 | **** | <0.0001 |
| TLR4 | hMSC-3d-OS vs. hMSC-21d-OS | -208.5 | -262.3 to -154.8 | **** | <0.0001 |
| | hMSC-3d-OSnano vs. hMSC-21d-OSnano | -125.7 | -177.9 to -73.52 | **** | <0.0001 |
| | hMSC-3d-OS150k vs. hMSC-21d-OS150k | -173.4 | -224.2 to -122.5 | **** | <0.0001 |
| | hMSC-7d-OS vs. hMSC-21d-OS | -223.1 | -275.2 to -170.9 | **** | <0.0001 |
| | hMSC-7d-OSnano vs. hMSC-21d-OSnano | -143.8 | -193.6 to -94.1 | **** | <0.0001 |
| | hMSC-7d-OS150k vs. hMSC-21d-OS150k | -176 | -229.7 to -122.2 | **** | <0.0001 |
| | hMSC-21d-con vs. hMSC-21d-OS | -220.1 | -269.9 to -170.4 | **** | <0.0001 |
| | hMSC-21d-con vs. hMSC-21d-OSnano | -141.1 | -190.8 to -91.34 | **** | <0.0001 |
| | hMSC-21d-con vs. hMSC-21d-OS150k | -175.4 | -225.1 to -125.6 | **** | <0.0001 |
| | hMSC-21d-OS vs. hMSC-21d-OSnano | 79.04 | 29.29 to 128.8 | **** | <0.0001 |
| | PDL-3d-OS vs. PDL-21d-OS | -43.91 | -52.72 to -35.09 | **** | <0.0001 |

Table 14 PCR positive results of HA receptors

| | | | | |
|---|---|---|---|---|
| PDL-3d-OSnano vs. PDL-21d-OSnano | -27.53 | -37.19 to -17.87 | **** | <0.0001 |
| PDL-3d-OS150k vs. PDL-21d-OS150k | -30.78 | -40.21 to -21.34 | **** | <0.0001 |
| PDL-7d-OS vs. PDL-21d-OS | -41.65 | -50.46 to -32.83 | **** | <0.0001 |
| PDL-7d-OSnano vs. PDL-21d-OSnano | -25.26 | -34.92 to -15.6 | **** | <0.0001 |
| PDL-7d-OS150k vs. PDL-21d-OS150k | -29.57 | -38.58 to -20.55 | **** | <0.0001 |
| PDL-21d-con vs. PDL-21d-OS | -37.68 | -46.49 to -28.86 | **** | <0.0001 |
| PDL-21d-con vs. PDL-21d-OSnano | -22.03 | -31.28 to -12.79 | **** | <0.0001 |
| PDL-21d-con vs. PDL-21d-OS150k | -24.95 | -33.96 to -15.93 | **** | <0.0001 |
| PDL-21d-OS vs. PDL-21d-OSnano | 15.64 | 6.396 to 24.89 | **** | <0.0001 |
| PDL-21d-OS vs. PDL-21d-OS150k | 12.73 | 3.715 to 21.74 | *** | 0.0004 |

Table 15 PCR positive results of OS markers

| Tukey's multiple comparisons test | | Mean Diff. | 95.00% CI of diff. | Significance | P Value |
|---|---|---|---|---|---|
| ALP | hMSC-3d-con vs. hMSC-3d-OS | -0.4212 | -0.7019 to -0.1405 | **** | <0.0001 |

Table 15 PCR positive results of OS markers

| | | | | |
|---|---|---|---|---|
| hMSC-3d-con vs. hMSC-3d-OSOSnano | -0.7119 | -0.9926 to -0.4312 | **** | <0.0001 |
| hMSC-3d-con vs. hMSC-3d-OS150k | -0.6258 | -0.9065 to -0.3451 | **** | <0.0001 |
| hMSC-3d-OS vs. hMSC-3d-OSnano | -0.2907 | -0.5714 to -0.009991 | * | 0.0348 |
| hMSC-3d-OS vs. hMSC-7d-OS | -0.5993 | -0.88 to -0.3186 | **** | <0.0001 |
| hMSC-3d-OSnano vs. hMSC-7d-OSnano | -0.6076 | -0.8883 to -0.327 | **** | <0.0001 |
| hMSC-3d-OS150k vs. hMSC-7d-OS150k | -0.6191 | -0.8998 to -0.3384 | **** | <0.0001 |
| hMSCVII-7d-con vs. hMSCVII-7d-os | -0.9495 | -1.23 to -0.6688 | **** | <0.0001 |
| hMSCVII-7d-con vs. hMSCVII-7d-OSnano | -1.249 | -1.529 to -0.9678 | **** | <0.0001 |
| hMSCVII-7d-con vs. hMSCVII-7d-OS150k | -1.174 | -1.455 to -0.8932 | **** | <0.0001 |
| hMSC-7d-OS vs. hMSC-7d-OSnano | -0.299 | -0.5797 to -0.01833 | * | 0.0254 |
| hMSC-7d-OS vs. hMSC-21d-OS | 0.8622 | 0.5483 to 1.176 | **** | <0.0001 |
| hMSC-7d-OSnano vs. hMSC-21d-OSnano | 0.8036 | 0.4898 to 1.117 | **** | <0.0001 |
| hMSC-7d-OS150k vs. hMSC-21d-OS150k | 0.3211 | 0.007269 to 0.6349 | * | 0.0396 |
| hMSC-21d-con vs. hMSC-21d-OS150k | -0.7011 | -1.045 to -0.3573 | **** | <0.0001 |
| hMSC-21d-OS vs. hMSC-21d-OSnano | -0.3576 | -0.7013 to -0.01378 | * | 0.0332 |

Table 15 PCR positive results of OS markers

| | | | | | |
|---|---|---|---|---|---|
| | hMSC-21d-OS vs. hMSC-21d-OS150k | -0.7654 | -1.109 to -0.4217 | **** | <0.0001 |
| | hMSC-21d-OSnano vs. hMSC-21d-OS150k | -0.4079 | -0.7516 to -0.06411 | ** | 0.0063 |
| | PDL-3d-OS vs. PDL-21d-OS | -1.049 | -1.429 to -0.6704 | **** | <0.0001 |
| | PDL-3d-OSnano vs. PDL-21d-OSnano | -1.752 | -2.131 to -1.373 | **** | <0.0001 |
| | PDL-3d-OS150k vs. PDL-21d-OS150k | -1.793 | -2.172 to -1.414 | **** | <0.0001 |
| | PDL-7d-OS vs. PDL-21d-OS | -1.072 | -1.451 to -0.6932 | **** | <0.0001 |
| | PDL-7d-OSnano vs. PDL-21d-OSnano | -1.774 | -2.153 to -1.395 | **** | <0.0001 |
| | PDL-7d-OS150k vs. PDL-21d-OS150k | -1.813 | -2.192 to -1.433 | **** | <0.0001 |
| | PDL-21d-con vs. PDL-21d-OS | -0.7852 | -1.2 to -0.37 | **** | <0.0001 |
| | PDL-21d-con vs. PDL-21d-OSnano | -1.488 | -1.903 to -1.072 | **** | <0.0001 |
| | PDL-21d-con vs. PDL-21d-OS150k | -1.529 | -1.945 to -1.114 | **** | <0.0001 |
| | PDL-21d-OS vs. PDL-21d-OSnano | -0.7023 | -1.117 to -0.2871 | **** | <0.0001 |
| | PDL-21d-OS vs. PDL-21d-OS150k | -0.7442 | -1.159 to -0.329 | **** | <0.0001 |
| BSP | hMSC-3d-OS vs. hMSC-7d-OS | -0.009754 | -0.01882 to -0.000692 | * | 0.0225 |
| | hMSC-3d-OS vs. hMSC-21d-OS | 0.01297 | 0.002986 to 0.02296 | ** | 0.0015 |

Table 15 PCR positive results of OS markers

| | | | | | |
|---|---|---|---|---|---|
| | hMSC-3d-OSnano vs. hMSC-21d-OSnano | 0.01323 | 0.003128 to 0.02333 | ** | 0.0013 |
| | hMSC-3d-OS150k vs. hMSC-7d-OS150k | -0.01876 | -0.02813 to -0.009391 | **** | <0.0001 |
| | hMSC-3d-OS150k vs. hMSC-21d-OS150k | 0.01152 | 0.001533 to 0.02151 | ** | 0.0093 |
| | hMSC-7d-con vs. hMSC-21d-con | 0.01268 | 0.002697 to 0.02267 | ** | 0.0022 |
| | hMSC-7d-OS vs. hMSC-21d-OS | 0.02273 | 0.01262 to 0.03283 | **** | <0.0001 |
| | hMSC-7d-OSnano vs. hMSC-7d-OS150k | -0.01466 | -0.02403 to -0.005297 | **** | <0.0001 |
| | hMSC-7d-OSnano vs. hMSC-21d-OSnano | 0.01658 | 0.006594 to 0.02657 | **** | <0.0001 |
| | hMSC-7d-OS150k vs. hMSC-21d-OS150k | 0.03028 | 0.0199 to 0.04066 | **** | <0.0001 |
| | PDL-3d-OSnano vs. PDL-7d-OSnano | -0.06066 | -0.1095 to -0.01181 | ** | 0.0031 |
| | PDL-7d-OS vs. PDL-21d-OS | 0.06611 | 0.01151 to 0.1207 | ** | 0.0046 |
| | PDL-7d-OSnano vs. PDL-21d-OSnano | 0.09944 | 0.04483 to 0.154 | **** | <0.0001 |
| | PDL-7d-OS150k vs. PDL-21d-OS150k | 0.08881 | 0.0342 to 0.1434 | **** | <0.0001 |
| COLLA1 | hMSC-3d-con vs. hMSC-3d-OSnano | -67.54 | -129.1 to -6.008 | * | 0.0178 |
| | hMSC-3d-OS vs. hMSC-3d-OSnano | -81.49 | -143 to -19.96 | ** | 0.001 |
| | hMSC-3d-OS vs. hMSC-21d-OS | 137.9 | 69.08 to 206.7 | **** | <0.0001 |
| | hMSC-3d-OSnano vs. hMSC-7d-OSnano | 88.07 | 26.54 to 149.6 | *** | 0.0002 |

Table 15 PCR positive results of OS markers

| | | | | |
|---|---|---|---|---|
| hMSC-3d-OSnano vs. hMSC-21d-OSnano | 219.9 | 151.1 to 288.7 | **** | <0.0001 |
| hMSC-3d-OS150k vs. hMSC-21d-OS150k | 177.8 | 109 to 246.6 | **** | <0.0001 |
| hMSC-7d-con vs. hMSC-21d-con | 77.48 | 8.691 to 146.3 | * | 0.0128 |
| hMSC-7d-OS vs. hMSC-21d-OS | 142 | 73.21 to 210.8 | **** | <0.0001 |
| hMSC-7d-OSnano vs. hMSC-21d-OSnano | 131.8 | 63.02 to 200.6 | **** | <0.0001 |
| hMSC-21d-con vs. hMSC-21d-OS | 83.18 | 7.819 to 158.5 | * | 0.0166 |
| hMSC-21d-con vs. hMSC-21d-OSnano | 83.71 | 8.348 to 159.1 | * | 0.0153 |
| hMSC-21d-con vs. hMSC-21d-OS150k | 83.12 | 7.763 to 158.5 | * | 0.0167 |
| PDL-3d-con vs. PDL-21d-con | -53.58 | -75.08 to -32.07 | **** | <0.0001 |
| PDL-3d-OSnano vs. PDL-21d-OSnano | -39.52 | -61.33 to -17.72 | **** | <0.0001 |
| PDL-3d-OS150k vs. PDL-21d-OS150k | -66.98 | -87.96 to -45.99 | **** | <0.0001 |
| PDL-7d-con vs. PDL-21d-con | -50.65 | -71.64 to -29.66 | **** | <0.0001 |
| PDL-7d-OSnano vs. PDL-21dOS-nano | -46.42 | -67.92 to -24.91 | **** | <0.0001 |
| PDL-7d-OS150k vs. PDL-21d-OS150k | -71.58 | -93.39 to -49.77 | **** | <0.0001 |
| PDL-21d-con vs. PDL-21d-OS | 45.05 | 22.07 to 68.04 | **** | <0.0001 |
| PDL-21d-OS vs. PDL-21d-OSnano | -27.51 | -50.49 to -4.516 | ** | 0.0056 |

Table 15 PCR positive results of OS markers

| | | | | | |
|---|---|---|---|---|---|
| | PDL-21d-OS vs. PDL-21d-OS150k | -53.7 | -76.68 to -30.71 | **** | <0.0001 |
| | PDL-21d-OSnano vs. PDL-21d-OS150k | -26.19 | -49.18 to -3.201 | * | 0.0111 |
| OCN | hMSC-3d-con vs. hMSC-21d-con | 0.1165 | 0.06398 to 0.169 | **** | <0.0001 |
| | hMSC-3d-OS vs. hMSC-3d-OSnano | -0.0599 | -0.1069 to -0.01291 | ** | 0.002 |
| | hMSC-3d-OSnano vs. hMSC-21dOS-nano | 0.1086 | 0.05607 to 0.1611 | **** | <0.0001 |
| | hMSC-3d-OS150k vs. hMSC-21d-OS150k | 0.05892 | 0.006385 to 0.1115 | * | 0.0136 |
| | hMSC-7d-con vs. hMSC-21d-con | 0.1422 | 0.08968 to 0.1947 | **** | <0.0001 |
| | hMSC-7d-OS vs. hMSC-7d-OSnano | -0.05842 | -0.1054 to -0.01143 | ** | 0.0031 |
| | hMSC-7d-OSnano vs. hMSC-21d-OSnano | 0.1211 | 0.06858 to 0.1736 | **** | <0.0001 |
| | hMSC-7d-OS150k vs. hMSC-21d-OS150k | 0.07555 | 0.02302 to 0.1281 | *** | 0.0002 |
| | PDL-3d-con vs. PDL-21d-con | -0.04057 | -0.0619 to -0.01925 | **** | <0.0001 |
| | PDL-3d-OS vs. PDL-7d-OS | -0.0498 | -0.06842 to -0.03119 | **** | <0.0001 |
| | PDL-3d-OSnano vs. PDL-7d-OSnano | -0.03744 | -0.05606 to -0.01883 | **** | <0.0001 |
| | PDL-3d-OS150k vs. PDL-7d-OS150k | -0.02814 | -0.04676 to -0.009527 | **** | <0.0001 |
| | PDL-7d-con vs. PDL-7d-OS | -0.03727 | -0.05646 to -0.01808 | **** | <0.0001 |

Table 15 PCR positive results of OS markers

| | | | | |
|---|---|---|---|---|
| | PDL-7d-con vs. PDL-21d-con | -0.03067 | -0.05199 to -0.009339 | *** | 0.0002 |
| | PDL-7d-OS vs. PDL-7d-OS150k | 0.01979 | 0.001174 to 0.03841 | * | 0.026 |
| | PDL-7d-OS vs. PDL-21d-OS | 0.03669 | 0.01588 to 0.05751 | **** | <0.0001 |
| | PDL-7d-OSnano vs. PDL-21d-OSnano | 0.03007 | 0.009251 to 0.05088 | *** | 0.0002 |
| | PDL-21d-con vs. PDL-21d-OS | 0.03009 | 0.007291 to 0.05289 | ** | 0.0011 |
| | PDL-21d-con vs. PDL-21d-OSnano | 0.04168 | 0.01887 to 0.06448 | **** | <0.0001 |
| | PDL-21d-con vs. PDL-21d-OS150k | 0.03282 | 0.01002 to 0.05562 | *** | 0.0002 |

Table 16 PCR positive results of cementogenic and ligamentogenic markers

| Tukey's multiple comparisons test | | Mean Diff. | 95.00% CI of diff. | Significance | P Value |
|---|---|---|---|---|---|
| CAP | hMSC-3d-con vs. hMSC-3d-os | -1.258 | -2.256 to -0.2596 | ** | 0.0025 |
| | hMSC-3d-OS vs. hMSC-3d-OS150k | 1.255 | 0.2567 to 2.253 | ** | 0.0026 |

Table 16 PCR positive results of cementogenic and ligamentogenic markers

| | | | | |
|---|---|---|---|---|
| hMSC-3d-OS150k vs. hMSC-7d-OS150k | -1.345 | -2.343 to -0.3474 | *** | 0.0008 |
| hMSCVII-7d-con vs. hMSCVII-7d-OS150k | -1.806 | -2.852 to -0.7589 | **** | <0.0001 |
| hMSC-7d-OS vs. hMSC-21d-OS | -1.516 | -2.645 to -0.3872 | *** | 0.0008 |
| hMSC-21d-con vs. hMSC-21d-OS | -2.731 | -3.981 to -1.482 | **** | <0.0001 |
| hMSC-21d-con vs. hMSC-21d-OSnano | -1.543 | -2.793 to -0.293 | ** | 0.0035 |
| hMSC-21d-con vs. hMSC-21d-OS150k | -1.734 | -2.984 to -0.4846 | *** | 0.0004 |
| PDL-3d-con vs. PDL-3d-OS | 3.12 | 0.5842 to 5.655 | ** | 0.0036 |
| PDL-3d-con vs. PDL-3d-OSnano | 3.787 | 1.252 to 6.322 | **** | <0.0001 |
| PDL-3d-con vs. PDL-3d-OS150k | 4.092 | 1.557 to 6.628 | **** | <0.0001 |
| PDL-3d-con vs. PDL-7d-con | -6.327 | -8.903 to -3.751 | **** | <0.0001 |
| PDL-3d-con vs. PDL-21d-con | 3.31 | 0.5164 to 6.104 | ** | 0.0064 |
| PDL-3d-OS vs. PDL-7d-OS | -11.22 | -13.76 to -8.688 | **** | <0.0001 |
| PDL-3d-OSnano vs. PDL-7d-OSnano | -8.215 | -10.83 to -5.604 | **** | <0.0001 |
| PDL-3d-OS150k vs. PDL-7d-OS150k | -11.56 | -14.13 to -8.985 | **** | <0.0001 |

Table 16 PCR positive results of cementogenic and ligamentogenic markers

| | | | | | |
|---|---|---|---|---|---|
| | PDL-7d-con vs. PDL-21d-con | 9.637 | 6.774 to 12.5 | **** | <0.0001 |
| | PDL-7d-OS vs. PDL-7d-OSnano | 3.676 | 1.1 to 6.252 | *** | 0.0002 |
| | PDL-7d-OSnano vs. PDL-7d-OS150k | -3.036 | -5.647 to -0.425 | ** | 0.0084 |
| | PDL-7d-OSnano vs. PDL-21d-OSnano | 10.61 | 7.744 to 13.47 | **** | <0.0001 |
| | PDL-7d-OS150k vs. PDL-21d-OS150k | 13.55 | 10.65 to 16.45 | **** | <0.0001 |
| CEM P1 | hMSC-3d-con vs. hMSC-21d-con | 0.1304 | 0.04575 to 0.2151 | **** | <0.0001 |
| | hMSC-3d-OS vs. hMSC-7d-OS | -0.07987 | -0.1556 to -0.004155 | * | 0.0284 |
| | hMSC-3d-OS vs. hMSC-21d-OS | -0.3851 | -0.4698 to -0.3005 | **** | <0.0001 |
| | hMSC-3d-OSnano vs. hMSC-21d-OSnano | -0.1626 | -0.2472 to -0.07793 | **** | <0.0001 |
| | hMSCVII-7d-con vs. hMSCVII-7d-OS | -0.156 | -0.2318 to -0.08031 | **** | <0.0001 |
| | hMSCVII-7d-con vs. hMSCVII-7d-OS150k | -0.1219 | -0.1976 to -0.04616 | **** | <0.0001 |
| | hMSC-3d-OS150k vs. hMSC-21d-OS150k | -0.2672 | -0.3519 to -0.1826 | **** | <0.0001 |
| | hMSC-7d-con vs. hMSC-21d-con | 0.08651 | 0.001851 to 0.1712 | * | 0.0401 |
| | hMSC-7d-OS vs. hMSC-7d-OSnano | 0.1234 | 0.04772 to 0.1992 | **** | <0.0001 |
| | hMSC-7d-OS vs. hMSC-21d-OS | -0.3053 | -0.3899 to -0.2206 | **** | <0.0001 |

Table 16 PCR positive results of cementogenic and ligamentogenic markers

| | | | | |
|---|---|---|---|---|
| hMSC-7d-OSnano vs. hMSC-7d-OS150k | -0.08928 | -0.165 to -0.01356 | ** | 0.0069 |
| hMSC-7d-OSnano vs. hMSC-21d-OSnano | -0.2351 | -0.3198 to -0.1505 | **** | <0.0001 |
| hMSC-7d-OS150k vs. hMSC-21d-OS150k | -0.2271 | -0.3118 to -0.1424 | **** | <0.0001 |
| hMSC-21d-con vs. hMSC-21d-OS | -0.5478 | -0.6405 to -0.4551 | **** | <0.0001 |
| hMSC-21d-con vs. hMSC-21d-OSnano | -0.3542 | -0.447 to -0.2615 | **** | <0.0001 |
| hMSC-21d-con vs. hMSC-21d-OS150k | -0.4355 | -0.5282 to -0.3427 | **** | <0.0001 |
| hMSC-21d-OS vs. hMSC-21d-OSnano | 0.1936 | 0.1008 to 0.2863 | **** | <0.0001 |
| hMSC-21d-OS vs. hMSC-21d-OS150k | 0.1123 | 0.01958 to 0.2051 | ** | 0.0046 |
| PDL-3d-con vs. PDL-7d-con | -1.043 | -1.523 to -0.563 | **** | <0.0001 |
| PDL-3d-OS vs. PDL-7d-OS | -1.537 | -2.017 to -1.057 | **** | <0.0001 |
| PDL-3d-OSnano vs. PDL-7d-OSnano | -0.7288 | -1.216 to -0.242 | **** | <0.0001 |
| PDL-3d-OS150k vs. PDL-7d-OS150k | -1.261 | -1.75 to -0.7725 | **** | <0.0001 |
| PDL-7d-con vs. PDL-7d-OSnano | 0.6718 | 0.1992 to 1.145 | *** | 0.0003 |
| PDL-7d-con vs. PDL-21d-con | 1.22 | 0.6993 to 1.741 | **** | <0.0001 |
| PDL-7d-OS vs. PDL-7d-OSnano | 1.007 | 0.5204 to 1.494 | **** | <0.0001 |

Table 16 PCR positive results of cementogenic and ligamentogenic markers

| | | | | | |
|---|---|---|---|---|---|
| | PDL-7d-OS vs. PDL-7d-OS150k | 0.5506 | 0.0483 to 1.053 | * | 0.0181 |
| | PDL-7d-OS vs. PDL-21d-OS | 1.794 | 1.26 to 2.327 | **** | <0.0001 |
| | PDL-7d-OSnano vs. PDL-21d-OSnano | 0.6848 | 0.1579 to 1.212 | ** | 0.0015 |
| | PDL-7d-OS150k vs. PDL-21d-OS150k | 1.254 | 0.7125 to 1.795 | **** | <0.0001 |
| SCX | hMSC-0d vs. hMSC-3d-con | -0.04928 | -0.09324 to -0.005315 | * | 0.0137 |
| | hMSC-3d-con vs. hMSC-3d-OSnano | 0.04288 | 0.002984 to 0.08277 | * | 0.0229 |
| | hMSC-3d-con vs. hMSC-7d-con | 0.05382 | 0.01329 to 0.09435 | ** | 0.001 |
| | hMSC-3d-con vs. hMSC-21d-con | 0.07535 | 0.03139 to 0.1193 | **** | <0.0001 |
| | hMSC-21d-con vs. hMSC-21d-OS | -0.04872 | -0.09687 to -0.0005608 | * | 0.0445 |

Table 17 One-way ANOVA of groups at day 3, 7 and 21

| | Sample | P value | P value summary |
|---|---|---|---|
| ALP | hMSC-ALP 3d | <0.0001 | **** |

Table 17 One-way ANOVA of groups at day 3, 7 and 21

| | | | |
|---|---|---|---|
| | hMSC-ALP 7d | <0.0001 | **** |
| | hMSC-ALP 21d | <0.0001 | **** |
| | PDL-ALP 3d | 0.0299 | * |
| | PDL-ALP 7d | <0.0001 | **** |
| | PDL-ALP 21d | <0.0001 | **** |
| BSP | hMSC-BSP 3d | 0.1995 | ns |
| | hMSC-BSP 7d | 0.0039 | ** |
| | hMSC-BSP 21d | 0.7851 | ns |
| | PDL-BSP 3d | 0.9097 | ns |
| | PDL-BSP 7d | 0.373 | ns |
| | PDL-BSP 21d | 0.0002 | *** |
| COL1A1 | hMSC-COL1A1 3d | 0.0095 | ** |
| | hMSC-COL1A17d | 0.2459 | ns |
| | hMSC-COL1A1 21d | <0.0001 | **** |
| | PDL-COL1A1 3d | 0.0007 | *** |
| | PDL-COL1A1 7d | <0.0001 | **** |
| | PDL-COL1A1 21d | 0.0016 | ** |
| OCN | hMSC-OCN 3d | 0.0051 | ** |
| | hMSC-OCN 7d | 0.0088 | ** |
| | hMSC-OCN 21d | <0.0001 | **** |
| | PDL-OCN 3d | 0.2154 | ns |
| | PDL-OCN 7d | <0.0001 | **** |
| | PDL-OCN 21d | 0.0009 | *** |

Table 17 One-way ANOVA of groups at day 3, 7 and 21

| | | | |
|---|---|---|---|
| SCX | hMSC-SCX 3d | 0.0087 | ** |
| | hMSC-SCX 7d | 0.3899 | ns |
| | hMSC-SCX 21d | <0.0001 | **** |
| | PDL-SCX 3d | 0.5003 | ns |
| | PDL-SCX 7d | 0.2043 | ns |
| | PDL-SCX 21d | 0.0021 | ** |
| CD44 | hMSC-CD44 3d | 0.0335 | * |
| | hMSC-CD44 7d | 0.3771 | ns |
| | hMSC-CD44 21d | <0.0001 | **** |
| | PDL-CD44 3d | <0.0001 | **** |
| | PDL-CD44 7d | 0.0009 | *** |
| | PDL-CD44 21d | <0.0001 | **** |
| CD168 | hMSC-CD168 3d | 0.093 | ns |
| | hMSC-CD168 7d | 0.0251 | * |
| | hMSC-CD168 21d | <0.0001 | **** |
| | PDL-CD168 3d | <0.0001 | **** |
| | PDL-CD168 7d | 0.0009 | *** |
| | PDL-CD168 21d | 0.3 | ns |
| TLR4 | hMSC-TLR4 3d | 0.0003 | *** |
| | hMSC-TLR4 7d | 0.0244 | * |
| | hMSC-TLR4 21d | <0.0001 | **** |
| | PDL-TLR4 3d | <0.0001 | **** |
| | PDL-TLR4 7d | <0.0001 | **** |
| | PDL-TLR4 21d | <0.0001 | **** |

Table 17 One-way ANOVA of groups at day 3, 7 and 21

| | | | |
|---|---|---|---|
| CAP | hMSC-CAP 3d | <0.0001 | **** |
| | hMSC-CAP 7d | 0.0002 | *** |
| | hMSC-CAP 21d | <0.0001 | **** |
| | PDL-CAP 3d | <0.0001 | **** |
| | PDL-CAP 7d | 0.014 | * |
| | PDL-CAP 21d | <0.0001 | **** |
| CEMP1 | hMSC-CEMP1 3d | 0.0653 | ns |
| | hMSC-CEMP1 7d | <0.0001 | **** |
| | hMSC-CEMP1 21d | <0.0001 | **** |
| | PDL-CEMP1 3d | 0.0002 | *** |
| | PDL-CEMP1 7d | 0.0003 | *** |
| | PDL-CEMP1 21d | <0.0001 | **** |

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