Elisa Mattias Sartori

Modulação da Expressão de miRNAs na Diferenciação de Osteoblastos em Nanosuperfícies

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Tese apresentada à Faculdade de Odontologia do Campus de Araçatuba - UNESP para obtenção do Título de "Doutora em Odontologia" – Área de concentração em Cirurgia e Traumatologia Buco-Maxilo-Facial.

Orientador: Prof. Dr. Osvaldo Magro Filho
Coorientador: Prof. Dr. Gustavo Mendonça

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Dedicatória

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"A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original."

Oliver Wendell Holmes Sr.

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RESUMO

Proposição: Este estudo teve como objetivo avaliar a modulação dos miRNAs que afetam o potencial osteogênico de CTMs em diferentes topografias de superfície de discos de vidro. **Material e Métodos:** Células tronco mesenquimais humanas foram plaqueadas nas diferentes superfícies e comparadas após 3, 7 e 14 dias para atividade de fosfatase alcalina, expressão de genes (Osteocalcina, Osteopontina, Sialo Proteína Óssea, Osterix, Runx2, BMP2 e ALP) e expressão de miRNAs. Microscopia eletrônica de varredura das superfícies com células foram obtidas para avaliação de adesão celular. **Resultados:** Atividade de fosfatase alcalina nas diferentes superfícies foi significantemente maior na superfície com nanotopografia. Do mesmo modo, a expressão de genes relacionados com osteoblastos foi mais elevada na superfície nano. Com 14 dias foi observado um aumento de 3.5 e 9 vezes para os genes Runx2 e Osterix, respectivamente. O gene da BMP2 e ALP também apresentou um aumento de 4 e 7 vezes comparado ao controle. Utilizando a tecnologia de sequenciamento de RNA (RNA-Seq) onde todos os RNAs existentes foram sequenciados, um total de 123 miRNAs com diferença de expressão foram encontrados comparando a superfície controle (dia 7) com a superfície nano (dia 14). 48 miRNAs apresentaram uma redução na expressão e 75 apresentaram um aumento de expressão. Alguns destes apresentaram marcadores para genes osteogênicos já identificados, tais como hsa-miR-135b-5p marcador para OCN, BSP, Runx2, CO15A1 e OSX, hsa-miR-122-5p marcador para OPN, hsa-miR-196a-5p marcador para BMP4, hsa-miR-26b-5p marcador para BMP2 e hsa-miR-148b-3p marcador para OPN. **Conclusão:** As superfícies com nanotopografia tem o potencial de melhorar a resposta de osseointegração de maneira a reduzir o tempo de osseointegração e também aumentar a produção de tecido ósseo ao redor dos implantes favorecendo assim áreas de qualidade óssea baixa. A utilização de miRNAs para alterar a resposta de diferenciação pode também ajudar a controlar o processo de osseointegração.

Palavras-chave: Biologia molecular. MicroRNAs. Propriedades de superfície. Materiais Biocompatíveis. Osteoblastos.

Sartori EM. Modulation of miRNA Expression in Osteoblast Differentiation in Nanosurfaces [thesis]. Araçatuba: UNESP – Sao Paulo State University; 2016.

ABSTRACT

Purpose: This study aimed to evaluate the modulation of miRNAs that affect the osteogenic potential of MSCs in different surface topographies of glass disks. **Material and Methods:** Human mesenchymal stem cells (hMSCs) were plated on different surfaces of glass disks and compared at 3,7 and 14 days for alkaline phosphatase (ALP) activity, expression of genes (Osteocalcin, Osteopontin, Bone Sialo Protein, Osterix, Runx2, BMP2 and ALP) and expression of miRNAs. Scanning electron microscopy of surfaces with cells were obtained to analyse the attachment of cells. **Results:** ALP activity on different surfaces was significantly greater in the nanotopography surface. At day 14 there was a 3.5-fold and a 9-fold increase for Runx2 and Osterix gene, respectively. BMP2 and ALP also increased by 4- and 7-fold compared to control. Using RNA sequencing technology (RNA-Seq) a total of 123 miRNAs were found differently expressed comparing control (day 7) to nano surface (day 14). 48 miRNAs were downregulated and 75 were upregulated. Some of them regulated osteogenic genes such as hsa-miR-135b-5p that targets OCN, BSP, RUNX2, CO15A1 and OSX, hsa-miR-122-5p wich targets OPN, hsa-miR-196a-5p targets BMP4, hsa-miR-26b-5p that targets BMP2 and hsa-miR-148b-3p that targets OPN. **Conclusion:** Surfaces with nanotopography have the potential to improve osseointegration response in order to reduce the time of osseointegration and also increase the production of bone tissue around the implants improving low bone quality areas. The use of miRNAs to affect differentiation response may also help control the osseointegration process.

Keywords: Molecular biology. MicroRNAs. Surface properties. Biocompatible Materials. Osteoblasts.

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Lista de Abreviaturas

ALP – fosfatase alcalina, alkaline phosphatase

BMP2- proteína óssea morfogenética 2, bone morphogenetic protein 2

BMPR1B - bone morphogenetic protein receptor type-1B

BSP - bone sialo protein

CFU-Obs - Colony forming units - Osteoblasts

COL4A3 - collagen type IV alpha 3

COL5A1 - collagen type V alpha 1

FOXN1 - forkhead box protein N1

hMSC –human mesenchymal stem cells

IGF1 - insulin-like growth factor 1

MAP2K7 - dual specificity mitogen-activated protein kinase kinase 7

MEV – Microscopia eletrônica de varredura

miRNAs – microRNAs

MSC – mesenchymal stem cells

NCOR2 - nuclear receptor corepressor 2

OCN – osteocalcina, osteocalcin

OPN – osteopontina, osteopontin

OSX – osterix

p-NPP - p-nitrophenol phosphate

PPP2CA - serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform

RIE - reactive ion etching

RUNX1 - runt-related transcription factor 1

RUNX2 - runt-related transcription factor 2

SCARB1 - scavenger receptor class B member 1

SEL1L - protein sel-1 homolog 1

SEM – Scanning electron microscope

SMAD2 - SMAD family member 2

SMAD4 - SMAD family member 4

VDR - calcitriol receptor

Sumário

Introduction

1 INTRODUCTION

Osseointegration is dependent upon the adhesion of a fibrin blood clot and the population of the implant surface by blood-derived cells and mesenchymal stem cells (MSCs) orchestrated in a manner that results in osteoid formation and its subsequent mineralization.[1] In select patient populations (e.g. smokers, diabetics, osteoporosis) some difficulty have been reported in achieving high rates of implant success.[2,3] The cause of these failures was largely attributed to a failure in bone formation in support of osseointegration. A major interest in understanding the cell-implant interaction is evident and clinical efforts to improve implant success have been focused on increasing the amount of bone that forms at the endosseous implant surface.

MSCs are multipotent cells that have the potential to self-renew and differentiate into various lineages, such as bone, cartilage, and muscle. miRNAs have been shown to mediate MSCs proliferation and differentiation.[4-6] Some miRNAs are directly involved in the formation of the human musculoskeletal system. miRNAs are expected to provide potential targets for the clinical treatment of metabolic bone diseases and bone healing (including the osseointegration process). The effects of miRNAs on bone biology regulation, with a particular focus on the miRNA-mediated control mechanisms of bone formation is a relative new field[7,8] and the effect of miRNA based on different titanium topographies has just began to be explored.[9,10] Thus, miRNAs have the potential to become a research focus for the osseointegration, especially for conditions such as osteoporosis and impaired bone healing.

In a previous study several miRNAs were down-regulated or up-regulated on modified titanium surfaces (microtopography) compared to smooth surfaces.[9] Some of these down-regulated miRNAs, such as miR-215, miR-125b and miR-20a can potentially target osteogenic genes like osterix (OSX), collagen type V alpha 1 (COL5A1), runt-related transcription factor 1 (RUNX1), forkhead box protein N1 (FOXN1), insulin-like growth factor 1 (IGF1), scavenger receptor class B member 1 (SCARB1), dual specificity mitogen-activated protein kinase kinase 7 (MAP2K7), calcitriol receptor (VDR), nuclear receptor corepressor 2 (NCOR2), bone morphogenetic protein receptor type-1B (BMPR1B), protein sel-1 homolog 1 (SEL1L), SMAD family member 2 (SMAD2), SMAD family member 4 (SMAD4), serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform

(PPP2CA), and collagen type IV alpha 3 (COL4A3). Several investigators have focused on studying the influence of nanotechnology on osteoblast-related behavior *in vitro* and *in vivo*.[11]

Nanotopography has direct and indirect effects on cell behavior. It mimics the cell environment favoring protein adsorption,[12] modulating cell/surface interactions and cell fate.[13,14] In previous gene expression studies[15-19] surfaces with nanotopography has repeatedly shown increase on mRNA levels for OSX, alkaline phosphatase (ALP) and bone sialo protein (BSP). The nanotopography has a strong influence on the differentiation of MSCs into osteoblasts. The miRNAs can play an important role in regulating the effect of different titanium surfaces on osteoblast differentiation. Because miRNAs participate in almost all cellular processes, data on the effects of miRNA on nanotopography titanium surfaces may help clarify the signaling network and demonstrate a regulatory effect even more precise and complex. These initial studies indicate a possible mechanism of translational repression and silencing of genes in the differentiation of MSCs into osteoblasts, which is affected by the nanotopography. However, it is still unclear how the cell responds to nanotopography signaling and how cell fate. This study aimed to evaluate the modulation of miRNAs that affect the osteogenic potential of MSCs in different surface topographies of glass disks.

Material and Methods

2 MATERIAL AND METHODS

2.1 Sample Surfaces Preparations and Surface Analysis

Samples surface preparation has been already described and is published in details elsewhere.[20] Briefly, photoresist was first spin-coated on glass wafers (Borofloat 33, Plan Optik, Elsoff, Germany) and patterned using photolithography to physically expose different regions of the underlying glass wafer. The patterned glass wafer was then processed with reactive ion etching (RIE) (LAM 9400, Lam Research, Fremont, CA) for different periods of time to generate different levels of the nanoscale surface roughness (R_q = 1–100 nm) on the exposed regions of the glass wafer, where the photoresist had previously been developed and dissolved. The RIE processing conditions used were: SF_6 (8 sccm), C_4F_8 (50 sccm), He (50 sccm), Ar (50 sccm), chamber pressure 1.33 Pa, bias voltage 100 V, and radio frequency power 500 W. The resulting RIE glass etch rate was about 100 nm min^{-1}. After the RIE process, photoresist was stripped using solvents, and the glass wafer was rinsed with distilled water (Figure 1). For unpatterned nanorough glass samples, bare glass wafers were directly processed with RIE using the same RIE conditions as described.[20] The glass wafers were cut into small pieces (1,5 cm × 1,5 cm) using a die saw (ADT7100; Advanced Dicing Technologies Ltd., Yokneam, Israel) before assays with cells.

The glass disks with 0 nm (control) and 100 nm (nano) were examined by high-resolution scanning electron microscopy (SEM) (Philips XL30 FEG, SEM, Philips, Eindhoven, Netherlands). The analysis and pictures were made at three randomly selected points on the surfaces of the disks of each group. The disks were placed in 70% alcohol for 15 minutes, after removal of the ethanol, placed 15 minutes in the ultraviolet light and kept in cold PBS (Gibco—Life Technologies, Grand Island, NY, USA) until use.

2.2 Cell Culture

Human mesenchymal stem cells (hMSCs) (Lonza Poietics ™, Portsmouth, NH, USA) were used to characterize the osteoblast differentiation in gene expression and miRNA profile during differentiation, and alkaline phosphatase activity. The cells were maintained and expanded in regular growth media wich consist of MEM Alpha modified with Earle salts

(MEM-α) (Gibco) supplemented with 10% FBS and antibiotics/antimycotic (penicillin/streptomycin/amphotericin B; Sigma Chemical Co, St Louis, MO) at 37° C and 5% CO_2. The osteogenic media consists of regular growth media supplemented with 10% FBS and antibiotic/antimycotic, and osteogenic supplements 10 mM glycerophosphate (Sigma) and 0.2 mM ascorbic acid (Sigma). This osteogenic medium was prepared without dexamethasone to avoid chemical induction of osteogenic differentiation. Rather a mechanical induction was intended.[13] 50,000 cells were plated in the glass disks (n = 3 for each surface). After 24 hours the osteogenic media was added to culture wells. This represented the initial time point (t = 0). The media was replaced every three days.

2.3 Scanning Electron Microscopy of Cell Adhesion

Twenty thousand cells were seeded on the glass surfaces and after 24 hours cells were fixed for SEM analysis. Samples were washed three times with cold PBS (Gibco), fixed for 1 hour with paraformaldehyde (Z-Fix), and dehydrated in a graded series of ethanol concentrations from 10% to 100% over a period of 1.5 hour. Dehydration in 100% ethanol was performed three times. Afterward dehydration, hexamethylsidilazane (Sigma) was added into the samples and let evaporate overnight under the hood. Samples were mounted on stubs, sputtered with gold palladium, observed, and photographed under a SEM microscope (Philips XL30 FEG, SEM, Philips).

2.4 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was measured at 3, 7 and 14 days. The wells with cells were washed 2X with cold PBS and lysed using 1% Triton X-100 (Sigma). The lysates were collected into 1.5 ml tubes and centrifuged at 14,000 rpm for 10 minutes. Only the supernatant collected was used for analysis. The ALP activity of cell lysates was assayed using 40 ul of each sample in a 96-well plate, added 200 ul of p-nitrophenol phosphate (p-NPP) (BluePhos® Microwell Phosphatase Substrate System. KPL, Gaithersburg, MD) and determined using a spectrophotometer (PowerWave HT, BioTek Instruments, Seattle, WA). ALP activity (units / ml) was normalized with total protein measurements for each sample

with wavelength at 595. Measurements of total proteins were performed using a Protein assay kit (Precision Red Advanced Protein Assay, Cytoskeleton INC, Denver, CO) and read in the spectrophotometer (PowerWave HT, BioTek Instruments) with wavelength at 630.

2.5 Analysis of Colony forming units - Osteoblasts (CFU-Obs)

The staining analyses were performed at 14 days with bone marrow cells collected by flushing from CD-1 mice (Charles River Laboratories, Boston, MA) femurs and tibiae. The project was approve by the University of Michigan Committee on Use and Care of Animals (UCUCA - protocolo PRO00006203). The femurs and tibiae were dissected away from attached muscle and connective tissue, the ends of the bones were removed, and marrow was extrud with a 21-gauge needle into the shaft of the bone and flushed it with 1ml of MEM-α supplemented with 10% FBS (Gibco). The media with the red blood cells were collected in 1.5 ml tubes and centrifuged, the supernatant were removed and the cell pellets were resuspended in fresh media. One million five hundred thousand bone marrow cells were plated on the Control and Nano glass disks in triplicate cultures. The non-adherent cell population was removed after 3 days and fresh media was added. The culture media from each well was aspirated and rinsed 2x with cold PBS. The rinse solutions were aspirated and the cells fixed with paraformaldehyde (Z-Fix) at room temperature for ten minutes. The Fixation Solutions were removed and the samples rinsed 2x with PBS. The Staining Solution (Alkaline Phosphatase Staining Kit, Sigma) was prepared by Mixing Solution A and Solution B at a 1:1 ratio, let it rest for 2 minutes, deionized water (ddH_2O) was added, and then Solution C. The PBS was aspirated and 1 ml of the prepared Staining Solution was added per well. The plates were covered with aluminum foil in the dark for approximately 1 hour. The staining solutions were removed by aspiration and washed with ddH_2O. Pictures were taken using a digital camera (Cannon A640 PowerShot, Japan).

2.6 RNA and miRNA Isolation for Gene Expression and miRNA Next Generation Sequencing

Total RNA was extracted at 7 and 14 days. Cells were washed 2X with cold PBS and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and collected by ethanol precipitation. Initial quantification of RNA was performed using the spectrophotometer (PowerWave HT, BioTek Instruments) and Gen5 (BioTek Instruments), followed by quality control evaluation in the Bioanalyzer (Agilent Technologies).

For mRNA analysis (gene expression), cDNA was synthesized using 500 ng of RNA following the manufacturer's recommendations (SuperScript VILO cDNA Synthesis, Invitrogen). The genes selected for gene expression analysis were: alkaline phosphatase (*alp*), bone morphogenetic protein 2 (*bmp 2*), bone sialo protein (*bsp*), osteocalcin (*ocn*), osteopontin (*opn*), osterix (*osx*) and runt-related transcription factor 2 (*runx2*). All primers were obtained from RT² qPCR™ Primer Assay (Qiagen Sciences, Germantown, MD, USA) used for real-time PCR. The reactions were prepared using the SYBR Green Real-Time PCR Master Mix (Qiagen) according to the manufacturer's protocol. ABI 7000 thermal cycler was used for real time PCR (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression was determined by $2^{-\Delta\Delta Ct}$ method and expressed as a relative expression level. As an internal control *GAPDH* gene (constitutive endogenous gene) was used.

For miRNA analysis using next-generation sequencing, miR-sequencing was performed in the Illumina HiSeq2000 (Illumina, San Diego, CA) using the latest versions of the sequencing reagents and flow cells providing up to 300 Gb of sequence information per flow cell. cDNA library preparation using NEBNext® Small RNA Library Prep Set for Illumina kits (NEB, Ipswich, MA) was done according to the manufacturer's instructions. Library construction consists of random fragmentation of the miR followed by cDNA production using random primers. Bioinformatics processing after sequencing included employ FASTQC to assess the overall quality of each sequenced sample, identify reads that may benefit from trimming or filtering, and clean the data as necessary. For RNA-Seq Bowtie/Tophat were used to align reads to the reference genome, and perform standard expression analysis using Cufflinks/Cuffdiff to identify differentially expressed transcripts between control samples and treatment samples. For miRNA analyses, after FastQC,

mirDeep2 software was used to identify known and novel miRNAs, and differential expression of miRNAs was done using EdgeR package. Gene targets that induce osteoblast differentiation were selected by bioinformatics from the top most potential targets listed in the Diana microT, TargetScan, or MIRANDA.

2.7 Data Analysis

Real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method and results reported as *fold change*. T-test was performed for comparison of mRNA levels compared to 0 nm (control) surfaces at day 7. The results of alkaline phosphatase activity were subjected to analysis of variance (ANOVA) and Fisher's test (LSD) when appropriate. For CFU-Obs T-test was performed. For all tests, results were considered significant if $p \leq 0.05$. The control group is characterized by 0 nm (control) surface disks at the earlier test time of each experiment.

Results

3 RESULTS

Surface and Cell Adhesion Analysis

Scanning electron microscope (SEM) images were taken to demonstrate the pattern surface of the samples (Figures 2 and 3) and also to confirm cell attachment. These surfaces have also been further characterized before by SEM[20]. Differences between the 0 nm (control) and the 100 nm (nano) were noted (Figures 2A-B and 3A-B). The nano surface showed a more roughened structure with nanofeatures measuring approximately 50 to 100 nm. (Figure 3B). hMSC cells were seeded in the glass disks and after 24 hours prepared for SEM analysis. At a small and a high magnification it is possible to observe presence of adherent cells onto the surfaces of the disks (Fig 2C-D and 3C-D).

ALP Activity and Colony Forming Unit-Osteoblasts

After 14 days of culture bone marrow cells on the disks were stained for alkaline phosphatase. It is possible to observe the formation of alkaline positive colonies on the disks samples (Figure 4). There were more colonies in the nano disks (Figure 4B) than in the control disks (Figure 4A). The quantification of alkaline phosphatase positive colonies demonstrated a statistically significant difference with an average of 4.3±3.2 colonies in the control disks and 13.3±3.2 colonies in the nano disks (Figure 4C).

For ALP activity there was no statistically significant difference between the two surfaces at day 3 (Figure 5). Alkaline phosphatase activity was statistically significant different between the control surface at 14 and 3 days (control). There was a statistically significant difference between the nano surfaces at 7 and 14 days comparing to the control surface.

Osteogenic Differention Gene Expression and miRNA Expression

For mRNA expression there was a 2.5- and 7-fold increase for ALP gene at 7 and 14 days, respectively, in the nano surfaces compared to control (0 nm - day 7). At day 14 the

increase between the control surface of ALP gene was 5.5-fold. BMP2 increased by 2- and 4-fold in the nano surface after 7 and 14 days, respectively. There was a 12.5-fold increase in the BSP gene in the nano surface with 7 days. At day 14 there was a 3.5-fold and a 9-fold increase for Runx2 and Osterix gene, respectively for nano surface (Figure 6).

Using RNA sequencing technology (RNA-Seq) a total of 123 miRNAs were differentially expressed comparing control (0 nm - day 7) to nano surface at day 14. Forty-eight miRNAs were down-regulated and 75 were up-regulated. When comparing the control with 7 and 14 days a total of 115 miRNAs were differentially expressed, 54 miRNAs were down regulated and 61 miRNAs were up-regulated. And when comparing control with the nano surface at day 7 a total of 10 miRNAs were differentially expressed, 3 miRNAs were down-regulated and 7 miRNAs were up-regulated (Tables 1 and 2).

Discussion

4 DISCUSSION

Recent studies demonstrated that local nanoroughness could regulate cell behavior such as cell morphology, proliferation, migration, differentiation and specific adhesion of different lineages of cells in different titanium surfaces.[9,11,13,16,18] The way these cells control and fate occurs for the formation of a new higher quality bone around dental implants surface are not yet well understood.[21] Attempts to improve the quality of osseointegration are frequent targets for molecular biology researches. The role that miRNAs play in these processes (i.e, cell differentiation, proliferation) could be one of the regulators of the effect of nanotopography on differentiation of osteoblastic cells.

Glass disks with a microfabrication strategy by the use of photolithography and RIE[20,22] were used in this study to facilitate production of large amount of samples for different experiments. This method also allowed the fabrication of different topographies that were initially tested (0, 50, 100, 200 and 400nm) (data not shown). SEM confirmed the patterned nanoroughness of the disks. The nano features of the rough surface were around 50 to 100 nm and are described elsewhere.[20] The hMSCs cells response could be noticed by the cell adhesion and proliferation on the control (0 nm) and nano (100 nm) glass surfaces. In other studies[23,24] hMSCs and MSC were platted on a polydimethylsiloxidane surface with nano-gratings and showed a significantly aligned cell cytoskeleton. The results of these findings could demonstrate that the nanopattern design in different surfaces, despite titanium, has significant effect in stem-cells behaviors as well.

In this study for the osteogenic media we did not add dexamethasone to drive osteoblast differentiation in any experiment. Dexamethasone is a well-known corticosteroid that can induce bone formation and is usually used *in vitro* for osteoblast differentiation.[13,16,18,24] Thereby leaving the surface acting unaided on osteoblast differentiation whereby other report[24] that demonstrated that nanoscale features could stimulate osteogenesis in the complete absence of osteogenic supplements.[24] And yet the nanorough glass surface tended to induce cell spontaneous differentiation.[20] In another experiment using mouse BMCs despite the absence of any osteogenic supplements, ALP staining as an indicator of osteoblast differentiation indicated that there were significantly more colonies grown on the nanotopography than on the smooth surface after 14 days. Also, higher ALP activity were observed after 7 and 14 days on the nano surface disks compared to

the control group (0 nm - day3). There is a positive correlation regarding alkaline phosphatase activity and the formation of osteoid tissue, which is a marker of osteoblastic differentiation.[25] On titanium disks with chemically produced nanotopography after 10 days analyses, it is reported[26] that there were more significantly reddish nodular regions than on control (smooth) and that after 14 days theses areas were larger and more abundant, using osteogenic cells collected from calvarial bone from Wistar rats.[26]

The relative gene expression of ALP, BMP2, BSP, OSX and RUNX2 had prominently increased on the nano surface compared to the control group (smooth - day7). This patterned of expression could be noticed on titanium micro and nanosurfaces that had been studied with osteoprogenitor cells[9,18,27,28] as well. The over expression of this osteo-related genes indicates that these changes could be attributed to the nanoscale surface used in this study. ALP is described as a marker of primary osteogenic activity and calcification regulator[29] and their presence proves the osteoblasts differentiation.[30] Runx2 and OSX are transcription factors essential for osteoblast differentiation and their increase are an indicative of osteoinductive and osteoblast differentiation.[16] BMP signaling pathways plays critical role in the regulation of osteoblast differentiation and it is described their potential and ability to control osteoblastic gene expression.[31] While BSP is related to the binding of basic elements in the extracellular bone matrix and bone mineralization,[32] and their presence could improve bone repair around dental implants.[33]

miRNAs execute their biological functions either by suppressing the translation or by deadenylation and subsequent degradation of various mRNA targets.[34] There are small noncoding RNAs molecules that play vital regulating functions on many physiological and pathological processes in the organism.[35] They can be detected in almost all cells in mammals, including cell death and/or proliferation on stem cells division, and so forth. Upon regulating the proliferation and differentiation of osteoblasts, they precisely control the activation or suppression of lots of osteo-related genes.[36] The miRNAs are not translated into proteins.[35] There are miRNAs that can affect several different genes, several of them related to osteogenesis.[37]

Next generation sequencing (RNA-Seq) for the miRNAs derived from hMSCs culture on the control and nano surface glass disks at different time points allowed us to perform an analysis of the profile of miRNAs expressed during the osteogenic differentiation controlled by nanotopography over time. This provided us primary information regarding the regulation

of translation induced by nanotopography. Comparing to hybridization methods, next-generation sequencing allows us to survey all available miRNAs at the samples and even novel miRNAs can be discovered using this technology.[28] Several miRNAs were found up- or down-regulated in this study at different time points and at both surfaces (Table 1 and 2). Interestingly the most up- or down-regulated miRNAs have not yet been linked to any osteogenic differentiation process. In this case hsa-miR-6082 was the most up-regulated miRNA at both day 7 (4.22 fold change) and day 14 (7.5 fold change) for nano surface. This miRNA did not show any expression changes at control surface at day 14 and appear to be driven by nanotopography. On the other hand, hsa-miR-202-5p, hsa-miR-204-5p and hsa-miR-205-5p appears to be a marker of osteogenic differentiation as it presented down-regulated in all groups at day 14 and did not have expression at day 7.

miRNA target analysis tools TargetScan 6.2 (http://www. targetscan.org) was used to explore potentials targets. Some of the most important miRNAs showed in this study were hsa-miR-135-5p, hsa-miR-196a-5p, hsa-miR-26a-5p, hsa-miR-148b-3p, and hsa-miR-122-5p. At day 14, three of them were down-regulated (>fivefold) compared the control surface at day 7, hsa-miR-196a-5p, hsa-miR-26b-5p and hsa-miR-148b-3p in the control and nano surfaces. These miRNAs target the BMP4, BMP2 and OPN genes, respectively. The other two miRNAs (hsa-miR-135b-5p and hsa-miR-122-5p) had an up-regulated response of on control and nano surfaces at day 14. They target the genes of OCN, BSP, RUNX2, COL15A1, and OSX, and OPN.[38,39]

Another interesting finding was that none of the miRNAs that had been previously reported as important in osteoblast differentiation had been found differentially expressed in our study (i.e., mir-31, mir-143) .[36,40-44] Also miRNAs that have been previously related to osteogenic differentiation on nanotopography did not present any differential expression in our study.[28,39] These differences could be related to a more late time point for harvesting the RNA. Nonetheless, the effects of these miRNAs need to be separately investigated to confirm their role in the osseointegration process. Other miRNAs can also be important in the osseointegration process and have related response to the nanotopography and need to be analyzed for a better understanding of their functions.

Conclusions

5 CONCLUSIONS

Within the limitations of this study we conclude that surfaces with nanotopography have the potential to improve osseointegration by directly affecting osteoblast differentiation in order to reduce the time of osseointegration and also increase the production of bone tissue around the implants. The expression of miRNAs is also an important factor that affects differentiation and may help control and understand the osseointegration process.

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Attachments

ATTACHMENT A - FIGURES

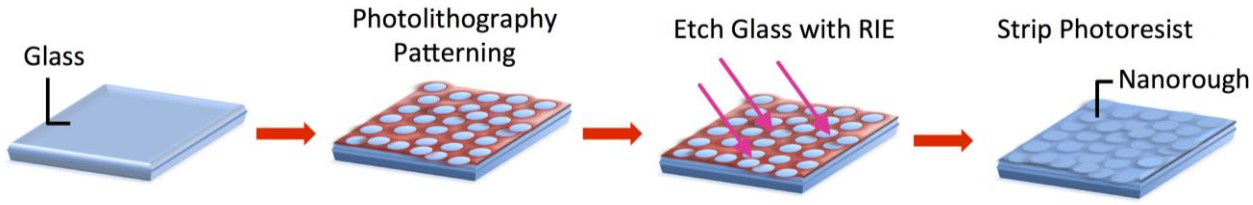

Figure 1 - Schematic of the fabrication process for patterned nanotopography glass substrates using photolithography followed by RIE.

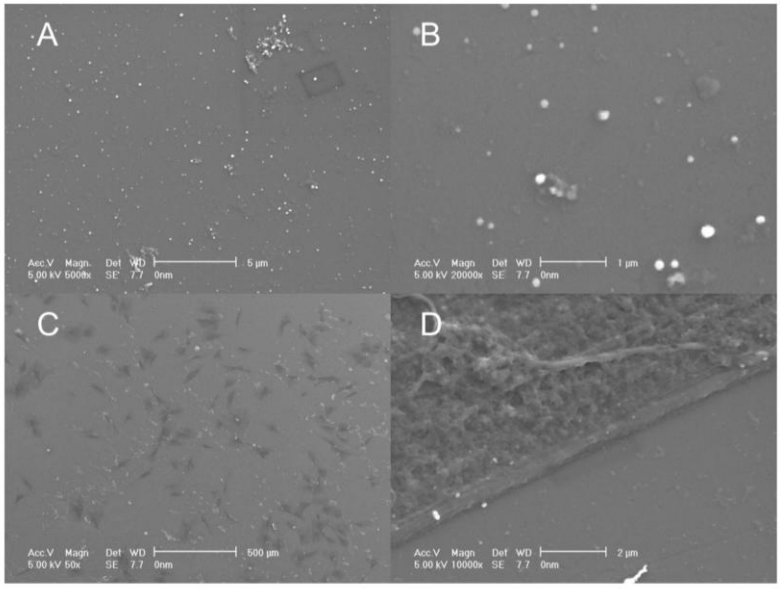

Figure 2 - Scanning Electronic Microscopy of the control surface. 5,000x (A) and 20,000x (B) magnification showing the surface topography. 50x (C) and 10,000x (D) magnification showing the presence of hMSCs on the surface.

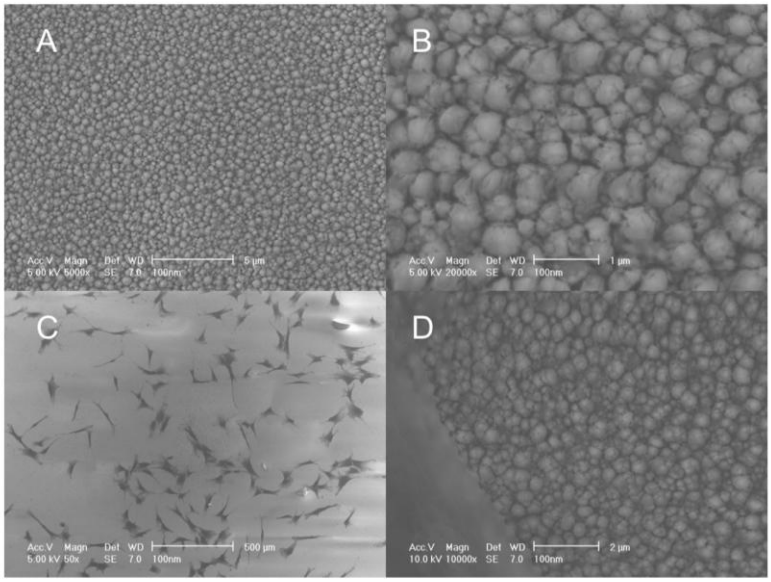

Figure 3 - Scanning Electronic Microscopy of the nano surface. 5,000x (A) and 20,000x (B) magnification showing the Nanotopography surface. 50x (C) and 10,000x (D) magnification showing the presence of hMSCs on the surface and interaction with the nanostructured surface.

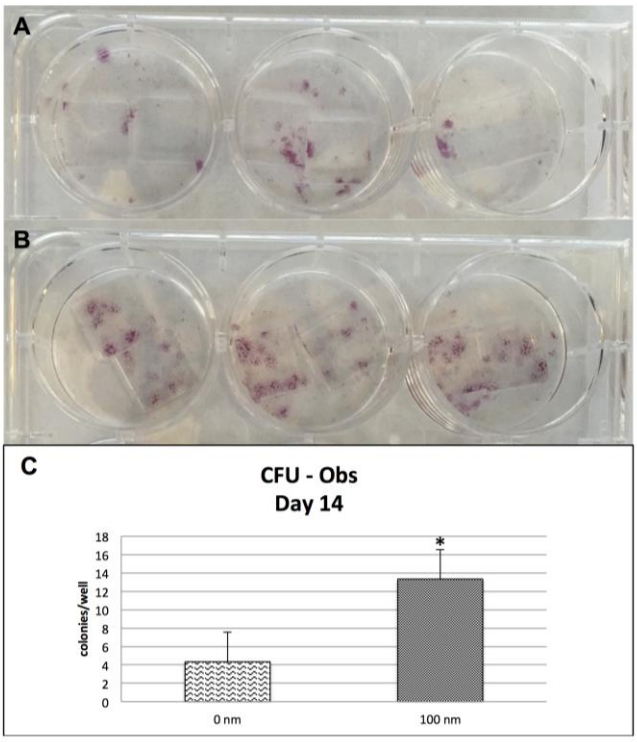

Figure 4 - Colony forming unit - Osteoblasts (CFU-Obs) formed on the Control (A) and Nano (B) surfaces after 14 days. (C) CFU-Obs quantification. Data shown as Mean±SD. n = 3. * Statistically significant difference ($p<0.05$).

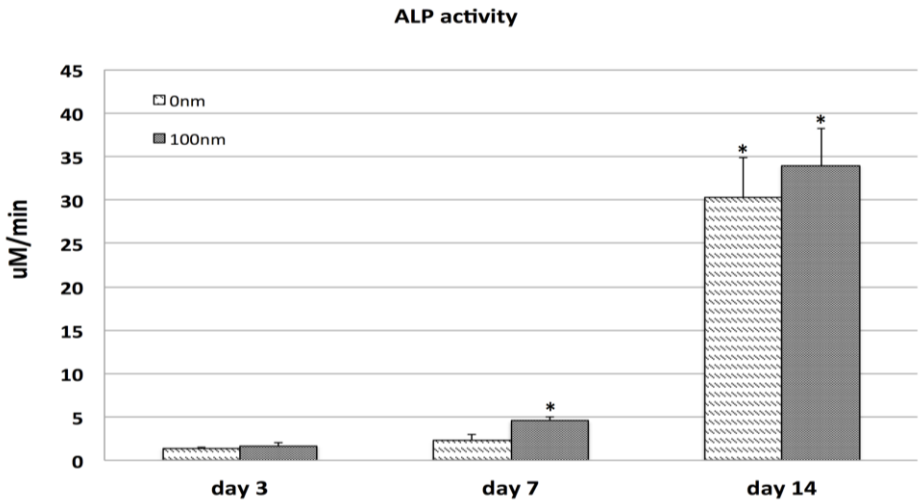

Figure 5 - Alkaline phosphatase activity in the control and nano surfaces. Alkaline phosphatase activity at 3, 7 and 14 days. Data shown as Mean±SD. n = 3; *p < 0.05.

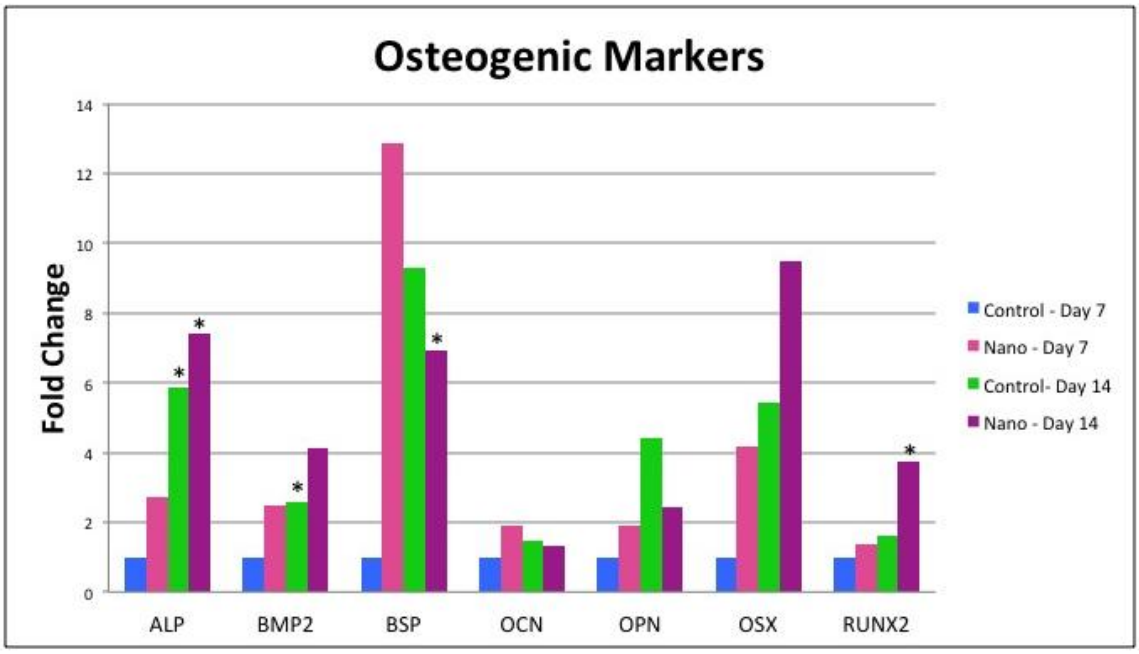

Figure 6 - Relative osteogenic gene expression level. Total RNA was isolated at 7 and 14 days. The results are shown in expression relative level to the control group - 0 nm at Day 7 (Method $2^{-\Delta\Delta Ct}$). n=3. GAPDH was used as housekeeping gene or endogenous control.

ATTACHMENT B - TABLES

Table 1 - miRNAs down-regulated on control at 14 days and nano surfaces at days 7 and 14 compared to control at 7 days (fold change, p value < 0.05).

miRNA ID	t-Test p Value	Fold Change Nano 7d	miRNA ID	t-Test p Value	Fold Change Control 14d	miRNA ID	t-Test p Value	Fold Change Nano 14d
hsa-miR-1469	0,0465	-2,213	hsa-miR-202-5p	0.00001	-6.564	hsa-miR-202-5p	0.00001	-4.554
hsa-miR-17-3p	0,0184	-1,966	hsa-miR-204-5p	0.00001	-5.935	hsa-miR-204-5p	0.00001	-3.855
hsa-miR-99a-3p	0,0179	-1,926	hsa-miR-205-5p	0.00001	-5.935	hsa-miR-205-5p	0.00001	-3.855
			hsa-miR-1469	0,0004	-3,857	hsa-miR-1469	0,0015	-3,390
			hsa-miR-664a-3p	0,0003	-3,082	hsa-miR-4428	0,0115	-3,068
			hsa-miR-199a-3p	0,0023	-2,551	hsa-miR-101-3p	0,0157	-2,888
			hsa-miR-199b-3p	0,0022	-2,551	hsa-miR-664a-3p	0,0011	-2,756
			hsa-miR-664a-5p	0,0004	-2,405	hsa-miR-3064-5p	0,0084	-2,483
			hsa-miR-300	0,0008	-2,403	hsa-miR-664a-5p	0,0015	-2,200
			hsa-miR-93-3p	0,0002	-2,319	hsa-miR-300	0,0042	-2,116
			hsa-miR-95-3p	0,0002	-2,319	hsa-miR-655-5p	0,0175	-2,103
			hsa-miR-374a-5p	0,0151	-2,065	hsa-miR-99a-3p	0,0083	-2,077
			hsa-miR-374b-5p	0,0151	-2,065	hsa-miR-199a-3p	0,0271	-1,965
			hsa-miR-374c-5p	0,0151	-2,064	hsa-miR-199b-3p	0,0269	-1,965
			hsa-miR-655-5p	0,0282	-2,013	hsa-miR-549a	0,0002	-1,949
			hsa-miR-26b-5p	0,0489	-1,970	hsa-miR-96-3p	0,0416	-1,942
			hsa-miR-196a-5p	0,0005	-1,919	hsa-miR-659-5p	0,0258	-1,940
			hsa-miR-1290	0,0211	-1,875	hsa-miR-199b-5p	0,0100	-1,855
			hsa-miR-1297	0,0223	-1,875	hsa-miR-652-5p	0,0356	-1,853
			hsa-miR-595	0,0161	-1,841	hsa-miR-148b-3p	0,0070	-1,826
			hsa-miR-598-3p	0,0161	-1,841	hsa-miR-146b-5p	0,0019	-1,783
			hsa-miR-599	0,0160	-1,841	hsa-miR-595	0,0304	-1,730
			hsa-miR-659-5p	0,0486	-1,820	hsa-miR-598-3p	0,0304	-1,730
			hsa-miR-30b-3p	0,0039	-1,802	hsa-miR-599	0,0302	-1,730
			hsa-miR-296-5p	0,0308	-1,799	hsa-miR-10b-5p	0,0052	-1,724
			hsa-miR-148b-3p	0,0087	-1,797	hsa-miR-26a-5p	0,0290	-1,653
			hsa-miR-30e-3p	0,0043	-1,796	hsa-miR-221-5p	0,0042	-1,619
			hsa-miR-26a-5p	0,0134	-1,768	hsa-miR-148a-3p	0,0341	-1,618
			hsa-miR-199b-5p	0,0176	-1,766	hsa-miR-223-5p	0,0044	-1,616
			hsa-miR-298	0,0358	-1,764	hsa-miR-33b-3p	0,0097	-1,607
			hsa-miR-103a-3p	0,0375	-1,755	hsa-miR-150-3p	0,0274	-1,603
			hsa-miR-146b-5p	0,0027	-1,745	hsa-miR-154-3p	0,0274	-1,603
			hsa-miR-29c-5p	0,0428	-1,721	hsa-miR-15a-3p	0,0273	-1,603
			hsa-miR-221-5p	0,0015	-1,710	hsa-miR-30a-3p	0,0036	-1,599
			hsa-miR-3929	0,0469	-1,708	hsa-miR-152-3p	0,0274	-1,599
			hsa-miR-3928-3p	0,0471	-1,708	hsa-miR-30d-3p	0,0039	-1,599
			hsa-miR-223-5p	0,0015	-1,707	hsa-miR-30b-3p	0,0226	-1,591
			hsa-miR-150-3p	0,0179	-1,660	hsa-miR-30e-3p	0,0249	-1,583
			hsa-miR-154-3p	0,0178	-1,660	hsa-miR-10a-5p	0,0066	-1,578
			hsa-miR-15a-3p	0,0178	-1,660	hsa-miR-99a-5p	0,0091	-1,531

hsa-miR-30d-3p	0,0019	-1,658	hsa-miR-196a-5p	0,0234	-1,526
hsa-miR-30a-3p	0,0018	-1,658	hsa-miR-335-3p	0,0158	-1,523
hsa-miR-152-3p	0,0178	-1,656	hsa-miR-339-3p	0,0160	-1,522
hsa-miR-1468-5p	0,0448	-1,646	hsa-miR-21-3p	0,0160	-1,502
hsa-miR-148b-5p	0,0402	-1,629	hsa-miR-340-3p	0,0500	-1,491
hsa-miR-10b-5p	0,0135	-1,617	hsa-miR-362-3p	0,0270	-1,447
hsa-miR-549a	0,0093	-1,590	hsa-miR-409-5p	0,0400	-1,421
hsa-miR-197-5p	0,0131	-1,582	hsa-miR-99b-5p	0,0460	-1,356
hsa-miR-23a-3p	0,0298	-1,556			
hsa-miR-211-3p	0,0408	-1,493			
hsa-miR-125a-5p	0,0274	-1,474			
hsa-miR-33b-3p	0,0343	-1,474			
hsa-miR-99a-5p	0,0179	-1,471			
hsa-miR-362-3p	0,0226	-1,465			
hsa-miR-214-3p	0,0291	-1,456			
hsa-miR-212-3p	0,0290	-1,456			
hsa-miR-193b-5p	0,0284	-1,448			
hsa-miR-210-3p	0,0315	-1,445			
hsa-miR-10a-5p	0,0318	-1,434			
hsa-miR-215-3p	0,0315	-1,432			
hsa-miR-24-1-5p	0,0486	-1,420			

Table 2 - miRNAs up-regulated on control at 14 days and nano surfaces at days 7 and 14 compared to control at 7 days (fold change, p value < 0.05).

miRNA ID	t-Test p Value	Fold Change Nano 7d	miRNA ID	t-Test p Value	Fold Change Control 14d	miRNA ID	t-Test p Value	Fold Change Nano 14d
hsa-miR-1273g-3p	0,0384	1,880	hsa-miR-409-3p	0,0267	1,466	hsa-miR-409-3p	0,0370	1,433
hsa-miR-4488	0,0268	1,943	hsa-miR-223-3p	0,0241	1,481	hsa-miR-223-3p	0,0266	1,471
hsa-miR-4481	0,0185	2,038	hsa-miR-222-3p	0,0244	1,481	hsa-miR-222-3p	0,0270	1,471
hsa-miR-1273h-5p	0,0372	2,083	hsa-miR-4529-5p	0,0210	1,514	hsa-miR-574-5p	0,0083	1,490
hsa-miR-138-2-3p	0,0064	2,289	hsa-miR-4521	0,0211	1,515	hsa-miR-570-5p	0,0083	1,490
hsa-miR-138-1-3p	0,0029	2,405	hsa-miR-612	0,0302	1,527	hsa-miR-579-5p	0,0082	1,490
hsa-miR-6082	0,0001	4,225	hsa-miR-586	0,0430	1,543	hsa-miR-548ai	0,0082	1,490
			hsa-miR-584-5p	0,0432	1,543	hsa-miR-619-3p	0,0346	1,525
			hsa-miR-615-5p	0,0269	1,544	hsa-miR-615-3p	0,0351	1,525
			hsa-miR-588	0,0434	1,545	hsa-miR-613	0,0355	1,525
			hsa-miR-587	0,0435	1,545	hsa-miR-616-3p	0,0362	1,529
			hsa-miR-583	0,0437	1,545	hsa-miR-4521	0,0159	1,543
			hsa-miR-581	0,0438	1,545	hsa-miR-612	0,0257	1,544
			hsa-miR-668-3p	0,0427	1,562	hsa-miR-4529-5p	0,0148	1,549
			hsa-miR-665	0,0383	1,579	hsa-miR-296-3p	0,0500	1,554
			hsa-miR-618	0,0221	1,580	hsa-miR-615-5p	0,0239	1,556
			hsa-miR-671-3p	0,0387	1,590	hsa-miR-671-3p	0,0475	1,559
			hsa-miR-617	0,0178	1,609	hsa-miR-610	0,0240	1,572
			hsa-miR-616-5p	0,0179	1,609	hsa-miR-616-5p	0,0230	1,576
			hsa-miR-610	0,0169	1,617	hsa-miR-617	0,0228	1,576
			hsa-miR-296-3p	0,0322	1,623	hsa-miR-618	0,0219	1,579
			hsa-miR-614	0,0119	1,692	hsa-miR-1226-5p	0,0496	1,637
			hsa-miR-619-3p	0,0079	1,702	hsa-miR-1228-5p	0,0370	1,701
			hsa-miR-615-3p	0,0080	1,702	hsa-miR-1225-5p	0,0343	1,711
			hsa-miR-613	0,0082	1,702	hsa-miR-1227-5p	0,0486	1,743
			hsa-miR-92b-5p	0,0241	1,704	hsa-miR-1224-5p	0,0350	1,802
			hsa-miR-616-3p	0,0080	1,713	hsa-miR-744-3p	0,0395	1,871
			hsa-miR-671-5p	0,0103	1,772	hsa-miR-4796-5p	0,0031	1,877
			hsa-miR-373-3p	0,0101	1,783	hsa-miR-4794	0,0030	1,877
			hsa-miR-377-3p	0,0101	1,783	hsa-miR-7704	0,0408	1,889
			hsa-miR-379-3p	0,0099	1,785	hsa-miR-7705	0,0409	1,889
			hsa-miR-372-3p	0,0080	1,807	hsa-miR-7702	0,0409	1,889
			hsa-miR-370-3p	0,0094	1,809	hsa-miR-5096	0,0388	1,904
			hsa-miR-486-3p	0,0003	1,911	hsa-miR-23a-5p	0,0112	1,929
			hsa-miR-485-3p	0,0003	1,911	hsa-miR-486-3p	0,0002	1,933
			hsa-miR-191-3p	0,0363	1,917	hsa-miR-485-3p	0,0002	1,933
			hsa-miR-483-3p	0,0002	1,944	hsa-miR-92a-1-5p	0,0226	1,938
			hsa-miR-135b-5p	0,0421	1,970	hsa-miR-92a-2-5p	0,0224	1,938
			hsa-miR-6082	0,0408	1,997	hsa-miR-1273g-3p	0,0293	1,945
			hsa-miR-5094	0,0302	2,030	hsa-miR-483-3p	0,0002	1,960
			hsa-miR-126-5p	0,0011	2,312	hsa-miR-5090	0,0352	1,966

hsa-miR-561-5p	0,0306	2,317	hsa-miR-5091	0,0354	1,966
hsa-miR-122-5p	0,0007	2,359	hsa-miR-4791	0,0011	1,972
hsa-miR-4489	0,0311	2,363	hsa-miR-4792	0,0009	1,997
hsa-miR-3940-3p	0,0370	2,413	hsa-miR-138-2-3p	0,0150	2,090
hsa-miR-3193	0,0225	2,519	hsa-miR-135b-5p	0,0260	2,090
hsa-miR-4488	0,0025	2,553	hsa-miR-3153	0,0418	2,183
hsa-miR-1273-5p	0,0100	2,591	hsa-miR-5094	0,0135	2,240
hsa-miR-7111-3p	0,0135	2,632	hsa-miR-138-1-3p	0,0047	2,314
hsa-miR-3687	0,0276	2,644	hsa-miR-323b-5p	0,0103	2,408
hsa-miR-1284	0,0188	2,696	hsa-miR-323a-5p	0,0102	2,408
hsa-miR-4486	0,0018	2,741	hsa-miR-4486	0,0044	2,472
hsa-miR-4481	0,0011	2,787	hsa-miR-3138	0,0106	2,555
hsa-miR-4467	0,0312	3,097	hsa-miR-3136-3p	0,0105	2,555
hsa-miR-4508	0,0002	3,108	hsa-miR-126-5p	0,0002	2,585
hsa-miR-4649-5p	0,0070	3,150	hsa-miR-122-5p	0,0002	2,612
hsa-miR-4505	0,0017	3,244	hsa-miR-4488	0,0017	2,629
hsa-miR-23c	0,0118	3,701	hsa-miR-4481	0,0008	2,837
hsa-miR-4459	0,0021	4,167	hsa-miR-7111-3p	0,0063	2,910
hsa-miR-4454	0,0023	4,172	hsa-miR-3197	0,0097	2,942
hsa-miR-4501	0,00009	4.370	hsa-miR-3189-3p	0,0095	3,089
hsa-miR-744-3p	0,00007	4.618	hsa-miR-3184-3p	0,0096	3,089
hsa-miR-7974	0,00005	5.036	hsa-miR-4489	0,0044	3,230
hsa-miR-4455	0,0006	5,112	hsa-miR-7974	0,0002	3,295
			hsa-miR-4459	0,0056	3,306
			hsa-miR-3187-3p	0,0058	3,418
			hsa-miR-4454	0,0030	3,814
			hsa-miR-4505	0,0001	4,397
			hsa-miR-4455	0,0008	4,540
			hsa-miR-6088	0,0002	4,674
			hsa-miR-6087	0,0002	4,674
			hsa-miR-6086	0,0002	4,674
			hsa-miR-4467	0,0012	6,511
			hsa-miR-3193	0,0009	6.882
			hsa-miR-6082	0,0001	7.484

ATTACHMENT C – UNIVERSITY OF MICHIGAN COMMITTEE ON USE AND CARE OF ANIMALS (UCUCA - PROTOCOL PRO00006203)

Principal Investigator: Gustavo Mendonca
Protocol: PRO00006203
Protocol Title: miRNA Modulation of Osteoblast Differentiation on Nanoscale Titanium Surfaces
Approval Period: 5/11/2015 - 5/11/2018

The University Committee on Use and Care of Animals (UCUCA) has reviewed your application to use vertebrate animals referenced below. This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals."

There may be additional issues that need to be addressed prior to initiation of the associated research. It is your responsibility, as Principal Investigator, to secure all the necessary requirements and recommendations (e.g., IBC approval, Animal Use training, accepting OSEH Findings and Recommendations).

When communicating with the UCUCA Office please refer to protocol PRO00006203 .

The approval date is 5/11/2015 . The approval period is for three years from this date. However, the United States Department of Agriculture (USDA) requires an annual review of protocols to use animals. Therefore, each year of this protocol prior to the anniversary of its approval date, you will be notified via email to submit a short annual review. Your continued animal use approval is contingent upon the completion and return of this annual review.

You will also be notified prior to expiration so that your renewal application can be prepared, submitted and reviewed in a timely manner to avoid noncompliance. For any change to the study, an amendment must be submitted to the UCUCA for review and approval prior to the implementation of the proposed change.

The University's Animal Welfare Assurance Number with the NIH Office Of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association For The Assessment And Accreditation Of Laboratory Animal Care International (AAALAC, Intl.) is November 6, 2009.

If you receive news media inquiries concerning any aspect of animal care or use in this project, please contact James Erickson, News and Information Services, 647-1842. If you have security concerns regarding the animals or animal facilities, contact Bill Bess, Director of Public Safety, 763-3434.

A formal approval letter will follow for each funding source listed on the protocol.

Sincerely,

Dr. Daniel D. Myers, DVM, MPH
Associate Professor and Chairperson
University Committee on Use and Care of Animals

ATTACHMENT D – Guidelines for Authors: *International Journal of Oral and Maxillofacial Implants*

Acceptable material.

Original articles are considered for publication on the condition they have not been published or submitted for publication elsewhere (except at the discretion of the editors). Articles on implant or tissue engineering (TE) basic or clinical research, clinical applications of implant/TE research and technology, proceedings of pertinent symposia or conferences, quality review papers, and matters of education related to the implant/TE field are invited.

Number of authors.

Authors listed in the byline should be limited to four. Secondary contributors can be acknowledged at the end of the article. (Special circumstances will be considered by the editorial chairman.)

Review/editing of manuscripts.

Manuscripts will be reviewed by the editorial chairman and will be subjected to blind review by the appropriate section editor and editorial staff consultants with expertise in the field that the article encompasses. The publisher reserves the right to edit accepted manuscripts to fit the space available and to ensure conciseness, clarity, and stylistic consistency, subject to the author's final approval.

Adherence to guidelines. Manuscripts that are not prepared in accordance with these guidelines will be returned to the author before review.

MANUSCRIPT PREPARATION

• The journal will follow as much as possible the recommendations of the International Committee of Medical Journal Editors (Vancouver Group) in regard to preparation of manuscripts and authorship (Uniform requirements for manuscripts submitted to biomedical journals.

Ann Intern Med 1997;126:36–47). See http://www.icmje.org

• Manuscripts should be double-spaced with at least a one-inch margin all around. Number all pages. Do not include author names as headers or footers on each page.

• Title page.

Page 1 should include the title of the article and the name, degrees, title, professional affiliation, and full address of all authors. Phone, fax, and e-mail address must also be provided for the corresponding author, who will be assumed to be the first-listed author unless otherwise noted. If the paper was presented before an organized group, the name of the organization, location, and date should be included.

• Abstract/key words.

The abstract should include a maximum of 350 words. A list of key words should be provided, not to exceed six. Abstracts for basic and clinical research articles must be structured with the following four sections: Purpose, Materials and Methods, Results, and Conclusions. Abstracts of short communications should also be structured but should be a maximum of 250 words. For all other types of articles (ie, literature reviews, technical and case reports), abstracts should not exceed 250 words and need not be structured.

• Article text.

Currently there is no article page limit (within reason).

• Acknowledgments.

Persons who have made substantive contributions to the study can be acknowledged at the end of the article. Also specify grant or other financial support, citing the name of the supporting organization and grant number.

• Legends.

Figure legends should be typed as a group at the end of the manuscript. Detailed legends are encouraged. For photomicrographs, specify original magnification and stain.

• Tables.

Each table should be logically organized, typed on a separate page at the end of the manuscript, and numbered consecutively. Table title and footnotes should be typed on the same page as the table.

• Abbreviations.

The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement.

• Trade names.

Generic terms are to be used whenever possible, but trade names and manufacturer name should be included parenthetically at first mention.

• Numbers.

Per SI convention, authors are requested to use decimal points rather than commas for fractional numbers.

REFERENCES

• All references must be cited in the text, numbered in order of appearance.

• The reference list should appear at the end of the article in numeric sequence.

• Do not include unpublished data or personal communications in the reference list. Cite such references parenthetically in the text and include a date.

• Avoid using abstracts as references.

• Provide complete information for each reference, including names of all authors (up to six). If the reference is to part of a book, also include title of the chapter and names of the book's editor(s).

Journal reference style:

1. Waasdorp J, Reynolds MA. Allogeneic bone onlay grafts for alveolar ridge aug- mentation: A systematic review. Int J Oral Maxillofac Implants 2010;25:525–531.

Book reference style:

1. Wikesjo UME, Hanisch O, Sigurdsson TJ, Caplanis N. Application of rhBMP-2 to alveolar and periodontal defects. In: Lynch SE, Genco RJ, Marx RE (eds). Tissue Engineering: Applications in Maxillofacial Surgery and Periodontics. Chicago: Quintessence, 1999:269–286.

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- Images saved in TIFF format are preferred, but JPG or EPS files are acceptable.
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- Images containing type should either be saved as a layered file or provided along with a second file with type removed.
- Line art (graphs, charts, drawings) should be provided as vector art (AI or EPS files)
- Please do not embed images into other types of documents (eg, Word, Excel, PowerPoint, etc).

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- Permissions and waivers should be faxed along with the Mandatory Submission Form to the JOMI Managing Editor (630-736-3634).

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