

**COMPORTAMENTO DE CÉLULAS ENDOTELIAIS AO
TITÂNIO CONTRIBUI PARA UM EFEITO PARÁCRINO
CAPAZ DE PROMOVER A DIFERENCIAÇÃO DE
OSTEOBLASTOS**

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“JÚLIO DE MESQUITA FILHO”

Instituto de Biociências de Botucatu

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“Se eu vi mais longe, foi por estar sentado sobre ombros de gigantes.”

Sir Isaac Newton

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LISTA DE ABREVIACÕES

ALP - Alkaline phosphatase

ANOVA - Análise de variância

BG - background correction

BSP - bone sialoprotein

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

cDNA - fita complementar de ácido desoxiribonucleico

CNPq - Conselho Nacional de Desenvolvimento Científico

Col1a1 – Cadeia alpha 1 de colágeno tipo 1

Col3a1 – Cadeia alpha 3 de colágeno tipo 1

CT - cycle threshold

Ctrl - control group

DAE - Dual Acid-Etched

DNA – Ácido desoxiribonucleico

DNMT1 - DNA methyltransferases 1

DNMT3A - DNA methyltransferases 3A

DNMT3B - DNA methyltransferases 3B

ECL - Enhanced chemiluminescence solution

ECM - extracellular matrix

ECs - endothelial cells

EDX - Energy Dispersive X-ray

eNOS - Endothelial nitric oxide synthase

FAK - Focal Adhesion Kinases

FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo

FBS - Fetal Bovine Serum

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GFAAS - Graphite furnace atomic absorption spectrometry

H2B – Histone 2B

HATs - histone acetyltransferase

HDAC1 - histone deacetylases 1

HDAC2 - histone deacetylases 2

HDAC3 - histone deacetylases 3

HDAC6 - histone deacetylases 6

HUVEC - Human Umbilical Vein Endothelial Cells

ISO – International Organization for Standardization
lnc-RNA – Long non-coding RNA
MMP - metalloproteinases
MMP2 - metalloproteinases 2
MMP9 - metalloproteinases 3
NO - Nitric Oxide
NOTCH – Neurogenic locus notch homolog protein
O.M. - Osteogenic Medium
OCN – osteocalcin
PCR - Polymerase Chain Reaction
pOb - pre-osteoblasts
PTP1B - protein tyrosine phosphatases 1B
PTPs - protein tyrosine phosphatases
qPCR - quantitative real time Polymerase Chain Reaction
RNA - Ribonucleic acid
ROS - reactive oxygen species
Runx2 - Runt-related transcription factor 2
SD - standard deviation
SEM - Scanning Electron Microscopy
SIN - Implants System Company
SIRT1 - NAD-dependent deacetylase sirtuin-1
SR - self-reverse system
Src - Receptor Tyrosine Kinase
SS – Static
TET - methylcytosine dioxygenase
TET1 - methylcytosine dioxygenase 1
TET2 - methylcytosine dioxygenase 2
TET3 - methylcytosine dioxygenase 3
Ti - Titanium
TIMP1 - metalloproteinase inhibitor 1
TIMP2 - metalloproteinase inhibitor 2
TiO₂ – Titanium oxide
W – with
Wo – without

RESUMO

Os mecanismos biológicos envolvidos com a osseointegração de implantes dentários a partir de ligas de titânio ainda não são totalmente conhecidos. Nosso grupo tem mostrado evidências importantes do ponto de vista do crosstalk entre osteoblastos e células endoteliais nesse processo. Para investigar se esse crosstalk estava ocorrendo nesse contexto, também consideramos a sinalização de células endoteliais relacionada à mecanotransdução sob tensão de cisalhamento laminar (até 3 dias), e esse meio enriquecido com fatores angiocrinos foi colhido posteriormente para ser usado na exposição de pré-osteoblastos (pOb) por até 7 dias in vitro. Dois tipos de superfícies de titânio foram considerados da seguinte forma: superfície com duplo ataque ácido (com DAE) e superfícies usinadas (sem DAE). Preliminarmente, mostramos que células endoteliais sofrem uma reprogramação epigenética em resposta ao meio enriquecido com titânio. Coletivamente, todos esses achados apoiam a hipótese de que o titânio mantém o microambiente circundante dinamicamente ativo e, portanto, afeta o desempenho das células endoteliais por meio da modulação epigenética. Especificamente, este estudo destaca a importância do HDAC6 como um participante desse processo, talvez correlacionado com a reorganização do citoesqueleto dessas células (Capítulo 2). Posteriormente, no Capítulo 3, mostramos que estas moléculas angiócrinas liberadas por células endoteliais são importantes no controle da diferenciação de osteoblastos: em resumo, nossos dados mostram a importância da mecanossinalização em favor da liberação de fatores bioativos pelas células endoteliais periimplante, que são responsáveis por criar um microambiente osteogênico capaz de induzir a diferenciação de osteoblastos e modular a remodelação da matriz extracelular (ECM). Levando isso em consideração, parece que as moléculas angiocrinas baseadas na mecanotransdução explicam o uso bem-sucedido do titânio durante a osseointegração.

ABSTRACT

The biological mechanisms involved in the osseointegration of dental implants made from titanium alloys are not yet fully understood. Our group has provided significant evidence from the perspective of crosstalk between osteoblasts and endothelial cells in this process. To investigate whether this crosstalk was occurring in this context, we also considered endothelial cell signaling related to mechanotransduction under laminar shear stress (up to 3 days), and this angiocrine-enriched medium was subsequently harvested for use in exposing pre-osteoblasts (pOb) for up to 7 days in vitro. Two types of titanium surfaces were considered as follows: double acid etching-treated surfaces (with DAE) and machined surfaces (without DAE). Preliminarily, we have shown that endothelial cells undergo epigenetic reprogramming in response to titanium-enriched medium. Collectively, all these findings support the hypothesis that titanium keeps the surrounding microenvironment dynamically active and thus affects the performance of endothelial cells by modulating epigenetics. Specifically, this study highlights the importance of HDAC6 as a participant in this process, perhaps correlated with the cytoskeleton rearrangement of these cells (Chapter 2). Subsequently, in Chapter 3, we demonstrate that these angiocrine molecules released by endothelial cells are crucial in controlling osteoblast differentiation. In summary, our data shows the relevance of mechanosignaling in favor of the release of bioactive factors by peri-implant endothelial cells, which are responsible for creating an osteogenic microenvironment capable of inducing osteoblast differentiation and modulating extracellular matrix (ECM) remodeling. Taking this into account, it appears that mechanotransduction-based angiocrine molecules explain the successful use of titanium during osseointegration.

CAPITULO 1

1. INTRODUÇÃO E MOTIVAÇÃO

Os biomateriais têm sido objeto de extensas pesquisas na área da saúde devido à sua importância na melhoria da qualidade de vida e na longevidade humana. Os biomateriais são dispositivos utilizados para substituir ou restaurar estruturas biológicas danificadas, como na recuperação de lesões ósseas. Devido ao aumento expressivo da expectativa de vida da população, a demanda por biomateriais tem crescido significativamente. Dentre as diversas opções de biomateriais, as ligas metálicas têm se destacado devido às suas características, como alta durabilidade, resistência à corrosão, estabilidade química, biocompatibilidade e baixa citotoxicidade (DE VITERI; FUENTES, 2013).

Em aplicações biomédicas que visam à recuperação de tecido ósseo, as ligas de titânio têm obtido altas taxas de sucesso e são amplamente recomendadas devido às suas excelentes propriedades mecânicas e químicas (BEZERRA et al., 2017; FERNANDES et al., 2018; ROSSI et al., 2017a). Além disso, essas ligas são eficazes na promoção da osteointegração, um processo relacionado principalmente à formação de uma camada de óxido em sua superfície, que estabelece um contato direto com o osso em processo de recuperação (DA COSTA FERNANDES et al., 2018a; DA S. FELTRAN et al., 2019; ROSSI et al., 2017b; SAGHIRI et al., 2016)

No entanto, contrariamente ao que se acreditava na ciência, implantes de titânio não são inertes e, portanto, causam respostas biológicas locais e sistêmicas (ALBANO et al., 2020; DA COSTA FERNANDES et al., 2018b; DA S. FELTRAN et al., 2019; DA SILVA et al., 2020; DE VITERI; FUENTES, 2013; FERREIRA et al., 2021; GOMES et al., 2020b). Nesse contexto, nosso interesse se volta para a análise da influência do implante de titânio no tecido vascular circundante, uma vez que o processo de regeneração óssea depende também de um suprimento sanguíneo adequado para fornecer nutrientes, oxigênio e células osteoprogenitoras (SAGHIRI et al., 2016). Esses mecanismos de cicatrização ao redor dos implantes envolvem uma série de eventos dinâmicos, nos quais a angiogênese desempenha um papel crucial, juntamente com a coagulação, inflamação e remodelação tecidual (AN et al., 2010). Além disso, a superfície do biomaterial de titânio libera partículas químicas em seu ambiente, que podem interferir

no metabolismo das células endoteliais que revestem o tecido vascular, afetando os mecanismos de transdução intracelular de sinais (SAGHIRI et al., 2014).

2. OSSO: ASPECTOS GERAIS

O tecido ósseo é altamente dinâmico e capaz de se remodelar ao longo de toda a vida. Além de fornecer suporte e proteção para os tecidos moles, o tecido ósseo atua como reserva de minerais, como cálcio, magnésio e fosfato, necessários para a manutenção de diversos processos fisiológicos (GEMINI-PIPERNI et al., 2014; ZAMBUZZI; MILANI; TETI, 2010). Devido à capacidade de armazenar e mobilizar esses íons minerais para o corpo, é necessária uma alta vascularização do tecido ósseo.

A cicatrização de lesões ósseas pode ocorrer por dois processos principais: reparação e regeneração. A regeneração ocorre quando o tecido danificado é repovoado com base na proliferação e diferenciação das células originais do tecido, resultando em tecido regenerado com propriedades funcionais e morfológicas semelhantes às do tecido original. Já no processo de reparação, o tecido danificado é substituído por um tecido conjuntivo fibroso, resultando em uma cicatriz de colágeno e na perda das características e funções originais. Atualmente, a regeneração óssea é vista como prioritária em casos de traumas, visando restaurar a forma e as funções originais do osso (ZAMBUZZI et al., 2011).

3. BIOMATERIAIS

Defeitos ósseos representam um dos principais desafios na área médica e odontológica. A reconstrução do tecido danificado é um dos maiores desafios para os cirurgiões, e muitas vezes é necessária a aplicação de biomateriais que permitam uma regeneração eficaz dos tecidos. Os materiais osteo-substitutos são uma das formas mais comuns e antigas de terapia regenerativa. Por exemplo, arqueólogos encontraram no Peru um crânio datado de 2000 a.C., onde uma placa de ouro de 1 mm de espessura cobria um defeito ósseo (HENKEL et al., 2013). Até o momento, nenhum material sintético foi desenvolvido que combine todas as propriedades do material autógeno, que é considerado o material ideal. Os materiais atualmente disponíveis têm limitações em termos de

estabilidade e retenção, o que pode levar a problemas como dificuldades na mastigação e dor devido ao deslocamento e soltura (SOHRABI et al., 2012).

A engenharia de tecidos para osso se baseia principalmente em três aspectos fundamentais: 1) recrutamento de células competentes, 2) moléculas de sinalização que estimulam as células para uma resposta eficaz e 3) matrizes de suporte que direcionam e sustentam a formação óssea durante a regeneração. Recentemente, a estabilidade mecânica também foi reconhecida como um quarto pilar essencial (CAFORIO; MANISCALCO, 2017; WEE et al., 2017). Estudos demonstraram que a estabilidade do microambiente onde ocorre a lesão influencia o sucesso da resposta biológica nos outros três aspectos (CAFORIO; MANISCALCO, 2017). Portanto, os biomateriais usados para regeneração óssea devem ser cuidadosamente projetados para fornecer suporte mecânico, criar uma matriz tridimensional adequada e promover a adesão e a proliferação celular.

4. ADESÃO DE CÉLULAS SOBRE MATERIAIS: PRIMEIRO PASSO INTEGRAÇÃO DE BIOMATERIAIS E REGENERAÇÃO ÓSSEA

A adesão celular desempenha um papel fundamental na regulação da diferenciação, proliferação e sobrevivência das células, sendo um aspecto amplamente estudado nos processos biológicos e patológicos (BARONCELLI et al., 2019; CAVAGIS et al., 2013; DA COSTA FERNANDES et al., 2018c; FERNANDES et al., 2014). Sua importância pode ser exemplificada pelo seu papel no desenvolvimento e na progressão do câncer, onde várias moléculas envolvidas na adesão celular promovem a invasibilidade e metástase tumoral. Recentemente, tem-se reconhecido a relevância do estudo da adesão celular em processos regenerativos e, especificamente, na integração de biomateriais para a regeneração óssea.

Para alcançar o sucesso na regeneração óssea, é desejável que os biomateriais induzam uma adesão celular eficaz. Nos últimos anos, nosso grupo de pesquisa concentrou esforços na busca por biomarcadores que elucidem a interação célula-material em biomateriais. Em um estudo recente, demonstramos que, nas primeiras 2 horas após o plaqueamento, o metabolismo das células osteoblásticas desencadeia uma série de vias de sinalização responsáveis pelo rearranjo do citoesqueleto. Isso envolve a ativação de proteínas como MAPKp38 e FAK (ZAMBUZZI et al., 2009). Além disso, nossos estudos também revelaram que a presença de Espécies Reativas de Oxigênio (EROs) modula a

ação de proteínas tirosina fosfatases (PTPs) durante os mecanismos celulares relacionados à adesão de osteoblastos (FERNANDES et al., 2014) (**Fig.1**).

Apesar dos avanços nesse campo, ainda há muito a ser descoberto sobre esses mecanismos em diversas condições, a fim de permitir a transferência de conhecimento para o desenvolvimento de novos materiais. A compreensão desses eventos é essencial para a melhoria de biomateriais utilizados na regeneração óssea e para o sucesso de intervenções cirúrgicas após a aplicação desses materiais.

Portanto, a adesão celular é um componente crítico na resposta do organismo à presença de biomateriais, e nosso estudo busca contribuir para a identificação de marcadores que revelem as propriedades adesivas de novos biomateriais, oferecendo uma alternativa valiosa aos métodos convencionais que frequentemente envolvem testes em animais. Isso é particularmente importante para promover avanços na área de métodos alternativos, visando uma melhor compreensão dos eventos celulares e moleculares desencadeados quando células endoteliais são expostas a meios enriquecidos com dispositivos à base de titânio, incluindo variações de superfície em escala micro e nanométrica já disponíveis no mercado brasileiro.

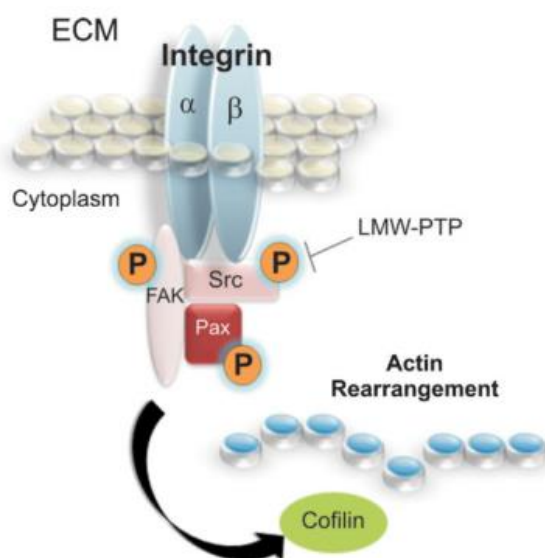


Figura 1. Mecanismos intracelulares decorrentes da adesão celular. Integrinas conectam o meio extracelular com o citoesqueleto da célula. Após captar sinais extracelulares as integrinas mobilizam moléculas de sinalização para o ponto de adesão focal. O balanço da fosforilação de FAK, Src e paxilina e, ao final desta cascata, de cofilina levará um sinal intracelular que será responsável pelo rearranjo dos filamentos

de actina. Estas alterações do citoesqueleto são responsáveis por adaptações celulares a superfícies diferentes. **Fonte:**(ZAMBUZZI et al., 2011).

5. ENDOTÉLIO E O FLUXO SANGUÍNEO

A pressão do fluxo sanguíneo realiza um importante papel na manutenção da homeostase vascular, onde as células endoteliais, que revestem a superfície luminal (**Figura 2**), são constantemente expostas às tensões mecânicas e, dentre elas, encontra-se o *shear-stress* ou Tensão de Cisalhamento, cuja característica é a força paralela e o componente tangencial de forças mecânicas no sentido do fluxo sanguíneo (**Figura 2**), originado pelas forças hemodinâmicas (DELA PAZ et al., 2012); esses mecanismos são importantes no tocante à liberação de fatores de relaxamento vascular, como o óxido nítrico (NO), desencadeando uma cascata de reações subjacentes às células musculares lisas vasculares por hiperpolarização, principalmente desencadeado por fatores hiperpolarizantes derivados do endotélio (WANG; KHALIL, 2018). O endotélio possui funções importantes na regulação da pressão sanguínea, bem como do suprimento sanguíneo nos tecidos, na angiogênese e remodelamento vascular, além de contribuir com resposta inflamatória e resposta imune do organismo (ANDO; YAMAMOTO, 2011).

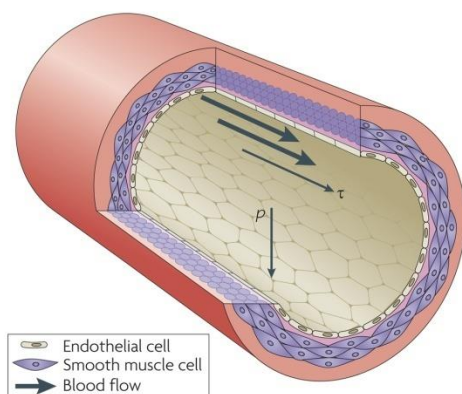


Figura 2. Tensões mecânicas exercidas na parede do vaso. O esquema do vaso sanguíneo ilustra a localização da célula endotelial (*endothelialcell*) e da célula muscular lisa (*smoothmusclecell*), bem como o sentido do fluxo sanguíneo (*bloodflow*) representado pela flecha mais grossa, e as tensões em que a pressão (p) é normal à parede do vaso, resultando em estiramento circunferencial da parede do vaso, e o *shear-stress* (τ) ou tensão de cisalhamento paralelo à parede do vaso e longitudinal no sentido do fluxo sanguíneo. **Fonte:** (HAHN; SCHWARTZ, 2009).

O endotélio compreende uma camada de células endoteliais que revestem todos os vasos sanguíneos, seja arterial ou venoso, e atuam de maneiras autócrina, parácrina e endócrina, as quais afetam o sistema cardiovascular com o intuito de realizar a manutenção da homeostase desse sistema (TRIGGLE; DING, 2011). Nas últimas décadas, estudos revelaram que, além do “Nitric Oxide” (NO), existem outros constituintes derivados do endotélio, assim como processos de regulação que também dependem do endotélio, que desempenham funções essenciais na manutenção do balanço que deve existir entre a vasoconstrição e vasodilatação, exemplos de vasoconstritores têm-se os peptídeos endotelina-1 e angiotensina II, e de vasodilatadores, além do NO produzido pela enzima Óxido Nítrico Sintase endotelial (eNOS), e a prostaciclina (TRIGGLE; DING, 2011).

Existem algumas atividades extremamente importantes do endotélio vascular como o desenvolvimento de novos vasos sanguíneos a partir de vasos existentes - um processo chamado de angiogênese – que ocorre no desenvolvimento normal, mas também ocorre em condições patológicas que envolvem reparo tecidual, regeneração de órgãos e formação e/ou aumento de tumores (MENTZER; KONERDING, 2014). A rede de vasos sanguíneos do sistema cardiovascular dos vertebrados fornece oxigênio e nutrientes para todos os tecidos, um minucioso processo necessário para atender suas altas demandas metabólicas (Hahn & Schwartz, 2009). Portanto as forças mecânicas que exercem tensões principalmente nas células endoteliais são importantes no sistema cardiovascular por agir com reguladores da fisiologia e patologia vascular (GIVENS; TZIMA, 2016).

A célula endotelial é influenciada diretamente pelo shear-stress e é capaz de se adaptar a essa tensão (SATHANOORI et al., 2017), além de perceber e responder diferencialmente aos padrões de fluxo exclusivos do seu microambiente, pois as mesmas possuem muitos mecanossensores que estão presentes na superfície apical, juncional e basal (GIVENS; TZIMA, 2016). O fluxo sanguíneo possui diferentes padrões que se enquadram em duas grandes categorias, o fluxo laminar (normal) e o fluxo perturbado (irregular), que são determinados pela geometria do vaso e dão origens à perfis de expressão gênica diferentes (GIVENS; TZIMA, 2016).

O fluxo sanguíneo irregular é algo inevitável em locais de ramificações do vaso (**Figura 3**) e o mesmo está relacionado com uma resposta inflamatória local que pode se agravar em determinadas classes de indivíduos (como diabéticos, fumantes, obesos e

sedentários) e progredir para a formação de placas ateroscleróticas (HAHN; SCHWARTZ, 2009), que é uma patologia cardiovascular, que pode debilitar o paciente e aumentar as chances de acidente vascular cerebral e infarto do miocárdio (GIVENS; TZIMA, 2016) e que pode ainda ser agravada por um quadro de pressão arterial elevada – a hipertensão (POULTER; PRABHAKARAN; CAULFIELD, 2015).

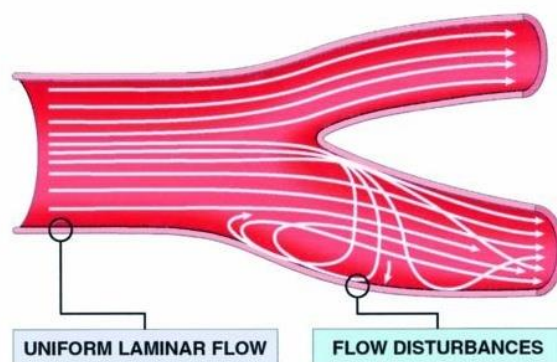


Figura 3. Fluxo sanguíneo laminar e fluxo sanguíneo perturbado. O desenho do vaso sanguíneo esclarece a diferença entre fluxo laminar regular uniforme do fluxo perturbado irregular, sendo este último geralmente situado em locais de bifurcações e associado com resposta inflamatória, o que aumenta a susceptibilidade a complicações cardiovasculares em determinadas circunstâncias específicas. **Fonte:** (COLLINS; CYBULSKY, 2001).

O sucesso de intervenções cirúrgicas após a aplicação biomédica de biomateriais depende da cicatrização bem-sucedida destas lesões. A cicatrização de feridas é um processo altamente complexo, composto por várias fases bem definidas, incluindo inflamação, epitelização, angiogênese e deposição de matriz. A angiogênese inadequada limita o transporte de nutrientes e gases entre a microvasculatura e os biomateriais implantados e sua regulação é baseada em numerosos fatores de crescimento, enzimas proteolíticas, componentes da matriz extracelular, moléculas de adesão celular e fatores vasoativos. Assim, nossos esforços serão concentrados para analisar respostas de células endoteliais ao meio enriquecido com titânio, a partir de um preparo de meio condicionado, como temos ganho experiência nos últimos anos (CÉLIO et al., 2021; DA C. FERNANDES et al., 2023; DA COSTA FERNANDES et al., 2021; FERNANDES et al., 2023; GOMES et al., 2020a, 2023; PINTO et al., 2022, 2023; RODRIGUES MARTINS et al., [s.d.]). Destaca-se ainda com este trabalho o fato de, a despeito de sua importância, ainda faltam alternativas à pesquisa com animais. Poucas são as metodologias de análise capazes de analisar e ranquear novos materiais a partir de ensaios

in vitro ou *in silico*. Consequentemente, universidades e companhias recorrem, quase sempre, a testes convencionais fazendo uso de modelos animais, comprometendo avanços necessários nesta área. Estamos convictos da contribuição deste projeto também na área de métodos alternativos, onde lançaremos os testes *in vitro* listados aqui como alternativa na busca de uma compreensão dos eventos celulares e moleculares envolvidos com células endoteliais desafiadas com o meio condicionado com dispositivos a base de titânio, com variações de superfície, em escala micro e nanométrica, já disponíveis no mercado brasileiro.

6. OBJETIVOS

Partindo desse contexto, o nosso objetivo geral foi investigar o comportamento de células endoteliais em resposta ao titânio e mapear sua capacidade de modular a atividade de genes em osteoblastos envolvidos com sua diferenciação. Especificamente pretendemos:

- Avaliar o efeito do meio condicionado por ligas de titânio na modulação epigenética de células endoteliais (Capítulo 2);
- Investigar se células endoteliais desempenham efeitos parácrinos em osteoblastos, através da análise de genes envolvidos com a diferenciação celular e remodelamento da matriz (Capítulo 3).

CAPITULO 2

Titanium-enriched medium promotes environmental-induced epigenetic machinery changes in human endothelial cells

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ABSTRACT

This is important to understand whether endothelial cells are epigenetically affected by titanium-enriched medium once angiogenesis is required during bone development and it is expected being recapitulated during osseointegration of biomaterials. To better address this issue, titanium-enriched medium was obtained from incubation of titanium discs up to 24 hours as recommended by ISO 10993-5:2016, and further used to expose human umbilical vein endothelial cells (HUVECs) up to 72 hours, when the samples were properly harvested to allow the molecular analysis related with epigenetics. In general, our data gathers an important repertoire of epigenetic players in endothelial cells responding to titanium, reinforcing protein related with the metabolism of acetyl and methyl groups, as follows: Histone deacetylases (HDACs) and NAD-dependent deacetylase sirtuin-1 (Sirt1), and DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) methylcytosine dioxygenases, which in conjunction culminates on driving chromatin condensation and methylation's profile of DNA strands, respectively. Taken our data into consideration, HDAC6 emerges as important player of this environment-induced epigenetic mechanism in endothelial cells, while Sirt1 being required in response to stimulation of ROS (reactive oxygen species) production, as its modulation with relevance on vasculature surrounding the implanted devices. Collectively, all these findings support the hypothesis that titanium maintains the surrounding microenvironment dynamically active and so affects the performance of endothelial cells by modulating epigenetics. Specifically, this study brings relevance to HDAC6 as a player of this process, maybe correlating with cytoskeleton rearrangement of those cells. Furthermore, as those enzymes are drugable, it opens new perspectives to consider the use of small molecules to modulate their activities as a biotechnological tool in order to improve angiogenesis and so accelerating bone growth with benefits on the fastest patients recovering time.

Keywords: Biomaterial; Titanium; Biological Analysis; Angiogenesis; Endothelial Cell; Epigenetic.

1. INTRODUCTION

The application of biomaterials has been widely proposed within the fields of tissue engineering and regenerative medicine, as well as to recover the functional performance in edentulism [1,2]. However, a significant number of patients may experience biological and mechanical complications characterized by surrounding tissue degradation and consequently to the failure of implant [3]. In an attempt to minimize the failure of implants, numerous researchers have endeavored to elucidate the physiological and molecular mechanisms devoted to the osteointegration mechanism [4–8]. In this sense, these biological data have guided the proposal of new surfaces presenting biomimetic properties predicted to develop necessary biological responses and so impacting the performance of surrounding host tissue during osteointegration [9,10].

Among the mostly used biomaterials within the biomedical area, titanium has been listed as the most usual metallic alloy in biomedical application. Indeed, titanium gathers well-accepted properties in biocompatibility scenario and it repercusses on the clinical success [11]. The biological performance of materials used in medicine and dentistry requires biological responses upon signaling related with to the interaction of cells and the physicochemical properties of their surfaces [12–17].

Among players related with regenerative tissue surrounding the implanted devices, there is a pleiotropic lineage of cells, which work together to promote new bone formation [18]. In this scenario, it is relevant to consider the coupling between endothelial cells and osteoblasts, which is known to compromise osteogenesis [19], [20]. Although a volume of knowledge has been improved considering osteoblasts responding to materials [21], the relevance of endothelial cells responding within this scenario is barely known [22–26]. During the biomaterials related osseointegration mechanism it is expected endothelial cell recapitulates the coupling with undifferentiated cells in order to optimize the osteogenesis and culminate in the success of the osseointegration. Although blood vessel is not properly in close contact with the implanted material's surfaces, we have recently reported that titanium-based devices release a considerable number of molecules and it interferes with cell metabolism away. Importantly, epigenetic mechanism drives the capacity of cells to respond the microenvironment changes. Epigenetics study the changes of gene expression without alterations of DNA itself, which include the reactions to modulate the incorporation of methyl groups into DNA strands as well as acetyl,

sumoyl, and phospho groups to histones [27]. Additionally, epigenetics regulating histone acetylation and DNA methylation are pivotal points involved in cellular responses during cell adhesion and viability [28]. Conversely, histone deacetylases (HDACs) affect gene expression in cells grown on implant surfaces [29]. Recently, differences in global DNA methylation profile were also demonstrated with relevance on genetic expression of osteogenic pathways in mesenchymal stem cells responding to titanium [30]. Generally, this mechanism has been shown present high performance with wound healing potential, ranging properties from high predictability to later cellular mechanism involved with osseointegration. In this way, we have reported earlier the biological responses of cell reacting to titanium and nanohydroxyapatite-blasted surfaces [31–33]. In this regard, tissue integration depends on biomaterial microstructure and composition, mainly considering the properties of the surfaces of materials, and it was better evaluated prospecting the effect of the pore size of titanium on the viability and function of fibroblasts and tenocytes in a dynamic bioreactor [34].

Again, the knowledge considering endothelial cells reacting to titanium alloys remains elusive although progress has been gathered considering osteoblasts [15,35–37]. Endothelial cells are enrolled with angiogenesis, which is essential to deliver nutrients and oxygen during the healing process surrounding biomaterial, as well as contributes with osteogenesis by coupling paracrine mechanism between osteoblasts and endothelial cells. To the best of our knowledge, this is the first study intending to explore the epigenetic machinery in endothelial exposed to titanium-enriched medium. Altogether, it involves epigenetic changes in human endothelial cells by processing genes and enzymes and these data are gathered to draw the complementarily molecular mechanism along wound healing repercussed by titanium-based devices in order to better predict the mechanisms involved with osseointegration of biomaterials into bone.

2. MATERIALS & METHODS

2.1. Reagents

The titanium discs were gently donated by S.I.N Implants System Company (SIN) (São Paulo, Brazil). Antibodies: HDAC1 (10E2) Mouse mAb #5356; HDAC2 (3F3) Mouse mAb #5113; HDAC3 (7G6C5) Mouse mAb #3949; HDAC6 (D2E5) Rabbit mAb #7558; SirT1 (1F3) Mouse mAb #8469; GAPDH (D16H11) XP® Rabbit mAb #5174; Life

Technologies/Molecular Probes, Inc. (Eugene, OR). DNMT1 Antibody (60B1220.1), DNMT3A Antibody (64B1446) (Novus Biologicals, LLC, USA), TET3 Antibody. Anti-DNMT3B (orb372330), TET1 (orb228563), and TET2 (orb131790) were purchased from Biorbyt (San Francisco, CA).

2.2. Titanium-enriched medium obtaining and experimental design

Titanium discs were previously prepared to subject their surfaces to dual acid-etched (DAE; w_Ti) and they were compared to machined surfaces (wo_Ti) [38]. Technically, to obtain the titanium-enriched medium from the samples, discs (0.6cm diameter) were incubated within conic tubes containing cell culture medium [0.2g/ml w/v, without Fetal Bovine Serum (FBS)] up to 24 hours as recommended by ISO 10993-5:2016 (**Fig.1a**). Thereafter, this titanium-enriched medium was later used to challenge human endothelial cells up to 72 hours, when the cells were harvested to allow the biological analysis in order to address the analysis of epigenetic mechanism (**Fig.1b**). **Fig.1b** depicts a draw of DNA methylation and histone acetylation molecular mechanisms. Additionally, both gene activation and repression are regulated by the pattern of acetylation of core histone, and the histone acetylation is mediated by coactivators (histone acetyltransferase: HATs) and corepressors (histone deacetylases: HDACs).

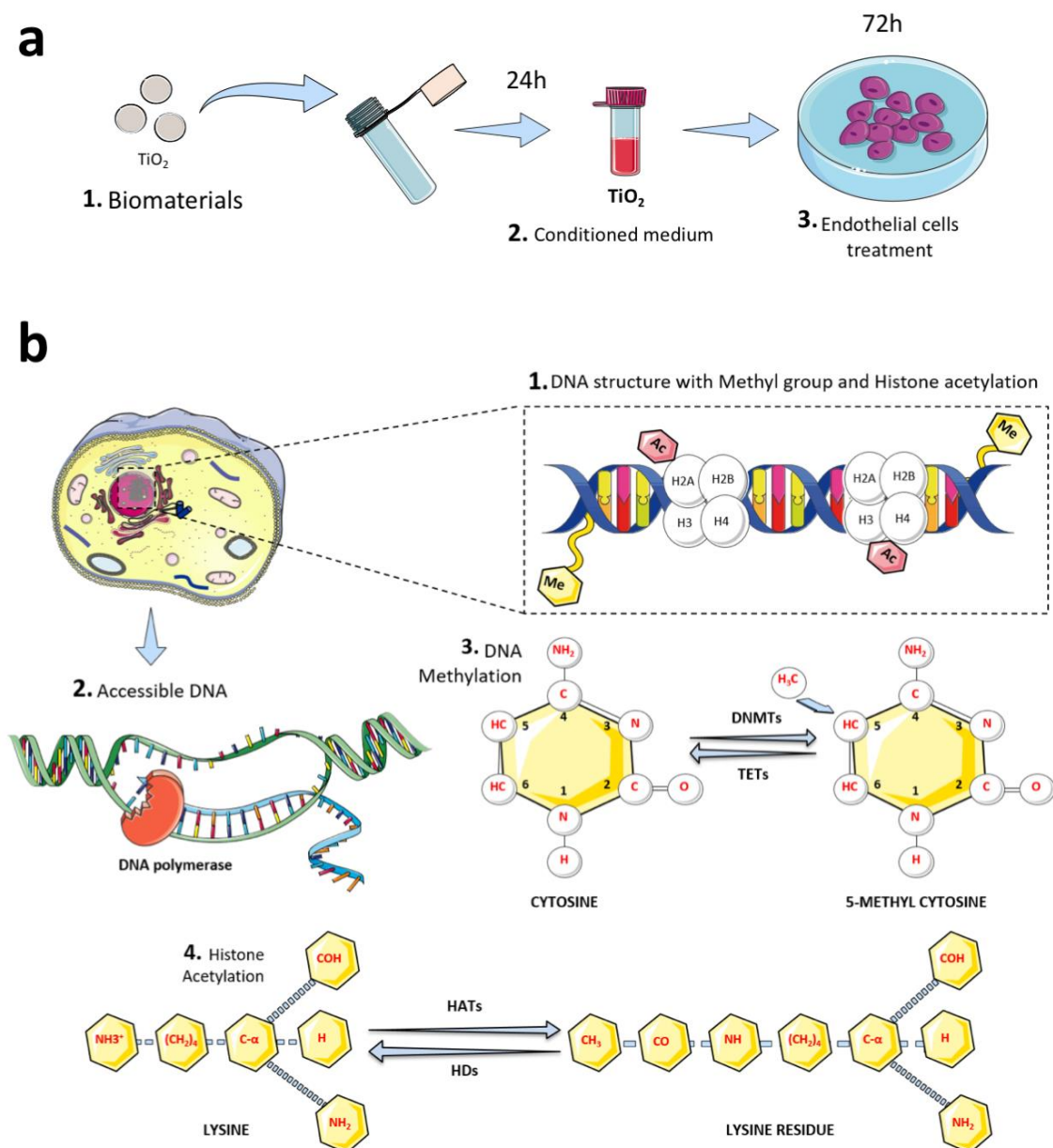


Fig.1. Experimental design performed in this study. **a)** In order to analyze whether Ti-enriched medium is able to modulate the epigenetic marks in endothelial cells, discs of titanium alloys were incubated into cell culture medium without FBS for up to 24 hours, as recommended by ISO 109993-5:2016. Thereafter, the conditioned medium (titanium-enriched medium) was used further to treat endothelial cells for up to 72 hours, when the cells were harvested to allow molecular analysis. The experiment was performed in triplicate (n=3); **b)** draw of the epigenetic machinery evaluated in this study: chromatin condensation by evaluating both gene expression and protein level of HDACs (4), and the metabolism of methyl moiety by evaluating DNMTs and TETs (1-3). The discs of titanium evaluated in this study was subjected to DAE (w_Ti) or not (wo_Ti).

2.3. Cell culture

To evaluate whether there are changes in endothelial cells responding to Ti-enriched medium and further correlating with aspects of angiogenesis, human umbilical vein endothelial cells (HUVECs; ATCC) were used in this study. Throughout the experiments, cells were grown in DMEM containing antibiotics (100U/ml penicillin, 100mg/ml streptomycin), and supplemented with 10% Bovine Fetal Serum (Nutricell, Campinas, SP, Brazil). Cells were maintained at 37°C, 5% CO₂, and 95% humidity. Important, these cells are mycoplasma-free and were used in this study in accordance with the manufacturer's recommendations.

2.4. Gene expression

RNA isolation and cDNA synthesis

Respecting the timeline, *the* total RNA was extracted using Ambion TRIzol Reagent (Life Sciences - Fisher Scientific Inc, Waltham, MA, USA) and its concentration and purity of total RNA determined by measuring the absorbance at 260/280 nm (NANODROP 2000, Thermo, MA, USA). After treatment of total RNA with DNase I (Invitrogen, Carlsbad, CA, USA), the cDNA was synthesized from 2 µg total RNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a standard 20 µL volume according to the protocol.

Gene expression

Gene expression in endothelial cells was measured using SYBR green quantitative PCR analysis. Real-time PCR amplification was performed with the *QuantStudio*® 3 Real-Time PCR (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). The 10 µL PCR system consisted of 1 µL cDNA (50 ng), 0.5 µmol forward primer, 0.5 µmol reverse primer, 5 µL SYBR green qPCR mix, and 3 µL ultrapure *DEPC*-treated Water. PCR primers were designed using Primer3 Input (version 0.4.0) software. The secondary structures and annealing temperatures analysis by Beacon Designer program, Free Edition (<http://www.premierbiosoft.com/>) and confirmed by the *in-silico* PCR

(<https://genome.ucsc.edu/>). Primer 5.0 software and, primers synthesis was acquired from Invitrogen Co. (Life Technologies). Primers sequences and CR conditions were presented in Table 1. Gene expressions were expressed as compared to control cells by $\Delta\Delta\text{CT}$ method, using *Gapdh* represented on the plate as housekeeping controls in three independent experiments in duplicate. Finally, cDNA was used to evaluate specific gene's expression (**Table 1**).

Table 1. Data sheet of the specific genes evaluated in this study.

Gene (ID)	Primer	5'- 3' Sequence	Reaction's Condition	Product size
HDAC1 (3065)	Forward	CTG GCC ATC ATC TCC TTG AT	95°C – 10s;	216 pb
			58°C – 30s;	
	Reverse	ACC AGA GAC GTG GAA ACT GG	72°C – 30s	
HDAC2 (3066)	Forward	TTC TCA GTG CAC CCA GTC AG	95°C – 10s;	170 pb
			59°C – 30s;	
	Reverse	CCA GTA TCC TTG GGG GAA AT	72°C – 30s	
HDAC3 (8841)	Forward	ACG TGG GCA ACT TCC ACT AC	95°C – 10s;	219 pb
			58°C – 30s;	
	Reverse	GAC TCT TGG TGA AGC CTT GC	72°C – 30s	
HDAC6 (10013)	Forward	AAG TAG GCA GAA CCC CCA GT	95°C – 10s;	416 pb
			59°C – 30s;	
	Reverse	GTG CTT CAG CCT CAA GGT TC	72°C – 30s	

SIRT (23411)	1	Forward	GCA GAT TAG TAG GCG GCT TG	95°C -15s; 60°C - 30s; 72°C - 30s	152 pb
		Reverse	TCT GGC ATG TCC CAC TAT CA		
DNMT1 (1786)		Forward	AGG ACC CAG ACA GAG AAG CA	95°C - 15s; 60°C - 30s; 72°C - 30s	201 pb
		Reverse	GTA CGG GAA TGC TGA GTG GT		
DNMT3A (1788)		Forward	AGG AAG CCC ATC CGG GTG CTA	95°C - 15s; 60°C - 30s; 72°C - 30s	225 pb
		Reverse	AGC GGT CCA CTT GGA TGC CC		
DNMT3B (1789)		Forward	TCG ACT TGG TGG TTA TTG TCT G	95°C - 15s; 60°C - 30s; 72°C - 30s	129 pb
		Reverse	TCG AGC TAC AAG ACT GCT TGG		
TET1 (80312)		Forward	GCC CCT CTT CAT TAC CAA GTC	95°C -15s; 60°C - 30s; 72°C - 30s	211 pb
		Reverse	CGC CAG TTG CTT ATC AAA ATC		
TET2 (54790)		Forward	GGT GCC TCT GGA GTG ACT GT	95°C -15s; 60°C - 30s; 72°C - 30s	245 pb
		Reverse	GGA AAA TGC AAG CCC TAT GA		

TET3 (200424)	Forward	GGT CAG GCT GGT TTA CAA CG	95°C -15s; 60°C - 30s; 72°C - 30s	198 pb		
	Reverse	GGC ATA GAC CCA CAC ACA TCT				
GAPDH (2597)	Forward	AAG GTG AAG GTC GGA GTC AA	95°C – 10s;	345 pb		
	Reverse	AAT GAA GGG GTC ATT GAT GG	58°C – 30s; 72°C – 30s			

2.5 Western blot

Cultures were exposed to titanium-enriched medium for up to 72 h, when the cells were properly lysed using lysis buffer [50 mM Tris-HCl, pH 7.4, 1% Tween 20, 0.25% Sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM O-Vanadate, 1 mM NaF, and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM aminoethylfluorosilicon 4-fluoride hydrochloride)] up to 2 hours in ice and thereafter sonicated, when the samples were properly centrifuged to collect the supernatant and discard the pellet. The protein concentration was determined by the Lowry method and an equal volume of Laemmli buffer [2X sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added. The obtained pool of proteins was boiled for 5 min at 95°C in a denaturant condition. To resolve the proteins into the gel, the proteins were resolved into SDS–PAGE (8% or 10%), when were transferred to PVDF membranes (Millipore), with sequential incubation with blocking solution containing 1% bovine serum albumin in Tris-buffered saline [TBST, Tween-20 (0.05%)] for 1 hour. Thereafter, the blocked membranes were subjected to specific primary antibody incubation for up to 12 hours, at 4°C. After, the membranes were extensively washed using TBS–Tween-20 (0.05%; 3x), when they were incubated with secondary antibodies for 1h at room temperature, when they were newly washed

using TBS. Enhanced chemiluminescence solution (ECL) was finally used to detect the bands. GAPDH was used as housekeeping and loading control.

2.6. Statistical Analysis

Bands of immunoblots were dimensioned using densitometry tools and analysis to obtain arbitrary values, which represent mean $\pm$ standard deviation (SD) in the graphs. Statistics analyses were applied using student's t-test (2-tailed) with $p < 0.05$ considered statistically significant and $p < 0.001$ considered highly significant. Accordingly, we have used one-way ANOVA with a post-test of Bonferroni, in order to compare all pairs of groups. In this case, the significance level was considered when $\alpha = 0.05$ (95% confidence interval). The software used was GraphPad Prism 6.

3. RESULTS

Epigenetic mechanism creates a bridge between the microenvironmental properties and capacity of activate genes. Specifically, we have focused on investigating the epigenetic-related mechanisms in endothelial cells responding to Ti-enriched medium by evaluating the profile of expression of enzymes related to (de)methylation of DNA and histone (de)acetylation. Thus, **Fig.2** brings the panorama of histone acetylation processes by exemplifying genes encoding main HDACs enzymes involved with the epigenetic mechanisms. The family of genes encoding HDACs in endothelial cells responding to Ti-enriched medium was evaluated, and our data show a significant reduction in gene expression of the HDAC1 (**Fig.2a,d**), HDAC2 (**Fig.2b,e**), and HDAC3 (**Fig.2c,f**) when compared to the control group. Importantly, the protein synthesis from those encoding genes presents a very similar profile to its respective transcript profile. Conversely, there is a significantly higher expression of HDAC6 gene in endothelial cells responding to w_Ti (**Fig.3a**), and it was corroborated with protein amount evaluation performed by western blot (**Fig.3c**), and it opens new perspectives to consider HDAC6 relevance on this scenario. Important to mention that SIRT1 was maintained lower in the cultures responding to both wo_Ti and w_Ti considering its transcript profile (**Fig.3b**), however, the protein seems to develop a constitutive effect in endothelial cells (**Fig.3d**). Importantly, a significantly higher expression of H2B was observed in response to both wo_Ti and w_Ti (**Fig.4**).

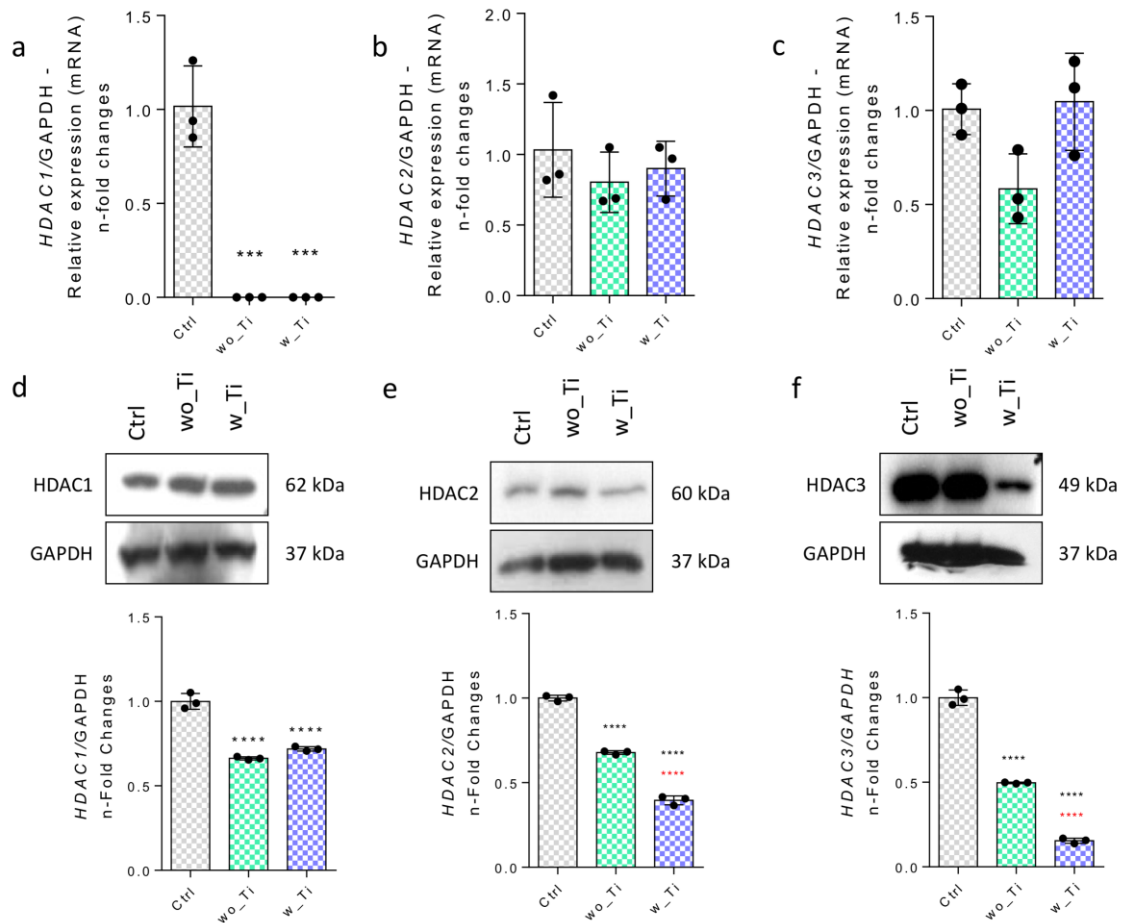


Fig.2. Effect of titanium-enriched medium on HDAC1, HDAC2 and HDAC3. The effect of Ti-enriched medium on the expression of genes related to histone acetylation HDAC1 (a), HDAC2 (b), HDAC3 (c) were evaluated by qPCR. The Control group (Ctrl) values was normalized to 1 and the relative values obtained to wo_Ti or w_Ti are shown in fold-changes. The effect was later evaluated regarding the protein level, and the profile was very similar to the transcripts. Note: HDACs: histones deacetylases; Ctrl: control group; wo_Ti: medium enriched with titanium without DAE; w_Ti: medium enriched with titanium with DAE. *** $p < 0.0002$, **** $p < 0.0001$ when compared with the control (Ctrl) group; and **** $p < 0.0001$ when compared to wo_Ti with w_Ti. The experiment was performed in triplicate (n=3).

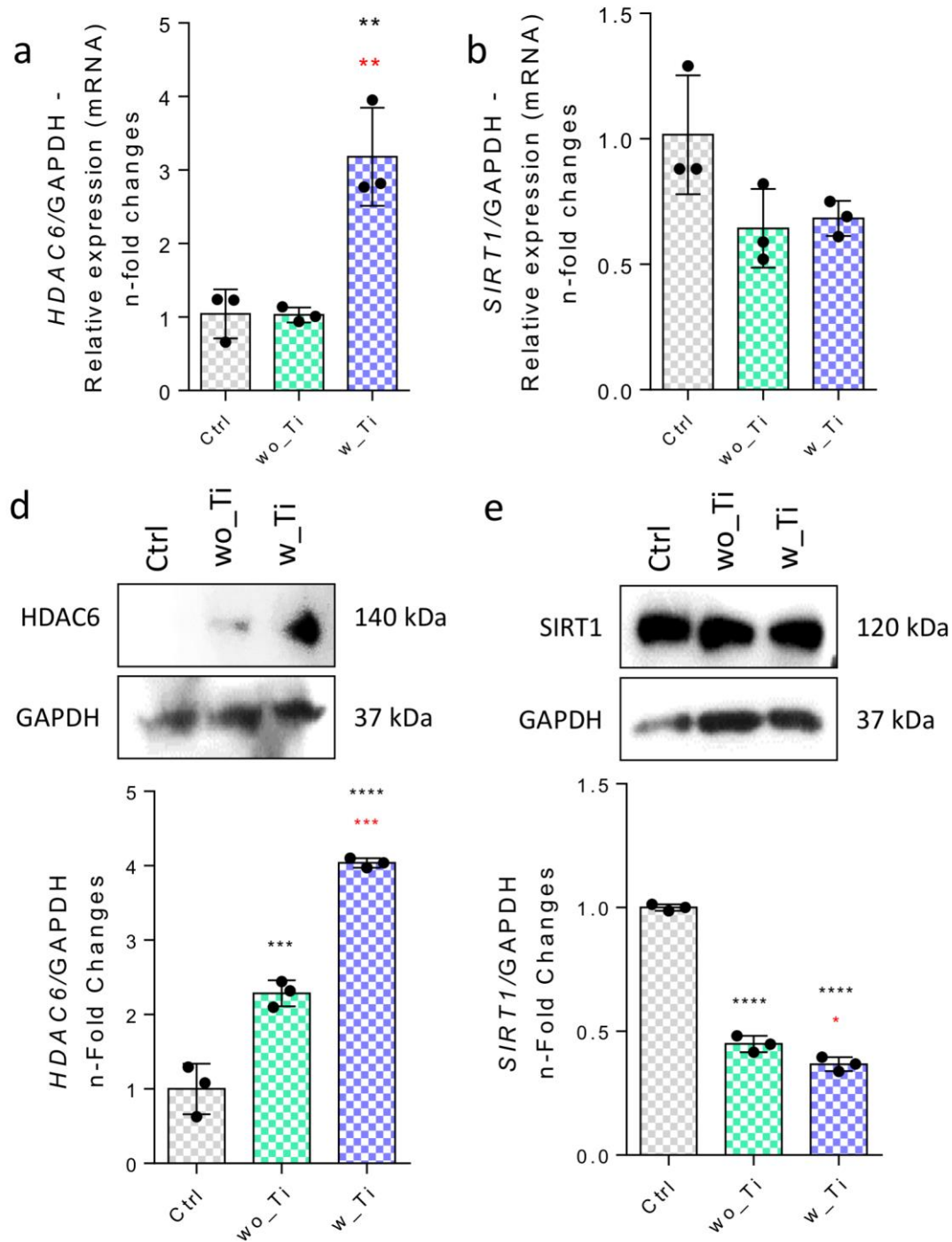


Fig.3. Effect of titanium-enriched medium on HDAC6 and SIRT1. The effect of Ti-enriched medium on the expression of HDAC6 (**a**) and SIRT1 (**b**) was evaluated by qPCR, and the protein expression by western blot was measured (**c,d**, respectively). Differences were considered statistically significant when $**p < 0.001$, $****p < 0.0001$ when compared with the control (Ctrl) group; and $*p < 0.04$, $**p < 0.001$, $***p < 0.0002$, when compared to wo_Ti with w_Ti. Note: HDACs: histones deacetylases, SIRT1:

Sirtuin 1, Ctrl: control group. GAPDH (gene and protein) was considered housekeeping gene in this study. The experiment was performed in triplicate (n=3).

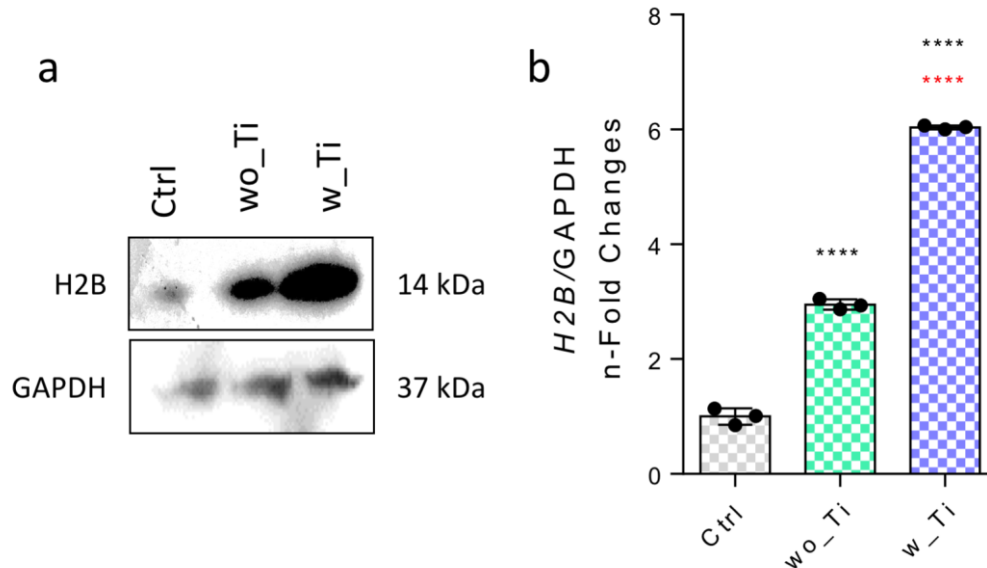


Fig.4. Effect of titanium-enriched medium on H2B. The effect of Ti-enriched medium on the expression of H2B was evaluated by performing western blot (a), densitometry and statistics is also shown (b). GAPDH was a loading control. ****p<0.0001 when compared with the control (Ctrl) group; and ****p<0.0001 when compared to wo_Ti with w_Ti. The experiment was performed in triplicate (n=3).

Thereafter, we have better addressed the involvement of epigenetic mechanisms displayed by DNA methyltransferases (DNMTs) and TETs, both related to regulating DNA methylation (**Fig.5f-j**). Our data shows DNMT1 gene was around 6-fold changes higher in response to w_Ti. than the control cultures (**Fig.5f**), while the both DNMT3A and DNMT3B genes were down-modulated (**Fig.5g,h**). However, this transcriptional profile doesn't repercuss to the protein synthesis once it was lower in endothelial cells responding to w_Ti (**Fig.5d**), while DNMT3A and 3B protein levels seem to respect the performance of those genes (**Fig.5e,f**).

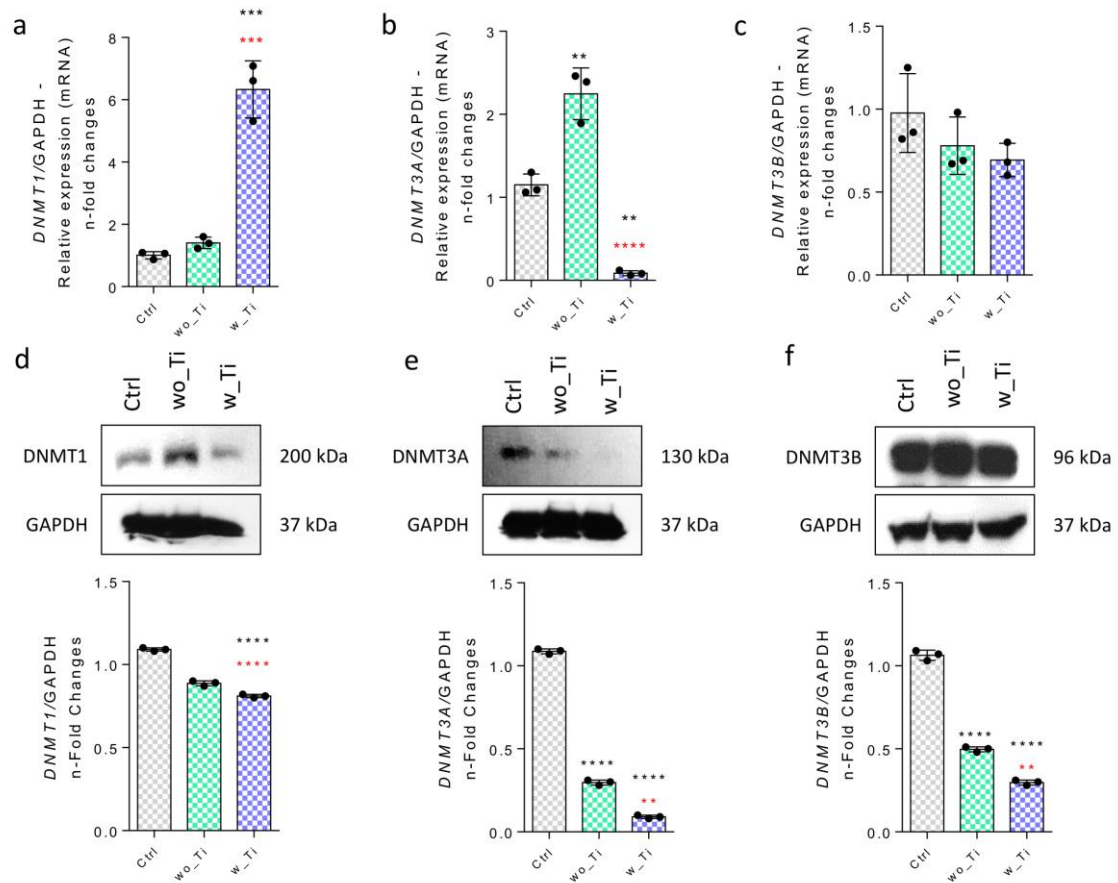


Fig.5. Effect of titanium-enriched medium on DNMTs. Firstly, the gene's activities were evaluated by qPCR, as follows: DNMT1 (a), DNMT3A (b), and DNMT3B (c), where GAPDH gene was used to normalize the data as a housekeeping gene. Thereafter, the protein profile was evaluated by western blot by resolving 75 μ g of sample per lane and later incubated with specific antibodies. A representative immunoblots of DNMT1 (d), DNMT3A (e), DNMT3B (f), are displayed. Densitometric analysis of immunoblots was normalized to the protein ratio of controls and GAPDH, here used as loading control (housekeeping control). Differences were considered statistically significant when $**p < 0.001$, $***p < 0.0002$, $****p < 0.0001$ when compared with the control (Ctrl) group; and $**p < 0.001$, $***p < 0.0002$, $****p < 0.0001$ when compared to wo_Ti with w_Ti. The graphs bring as mean $\pm$ standard deviation of three independent experiments. DNMT: DNA methyltransferase, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, Ctrl: control group; wo_Ti: medium enriched with titanium without DAE; w_Ti: medium enriched with titanium with DAE. The experiment was performed in triplicate (n=3).

Also, we investigated both TET1 and TET2 genes and related proteins by using qPCR and western blot technologies respectively. TET1 and TET3 genes remain unchanged (**Fig.6a,c**) while the TET2 gene was higher (~ 2.5 -fold changes) than the control cultures in endothelial cells responding to both wo_Ti and w_Ti (**Fig.6b**), although protein content shows a significant decrease of both TET1 and 2 (**Fig.6d,e**).

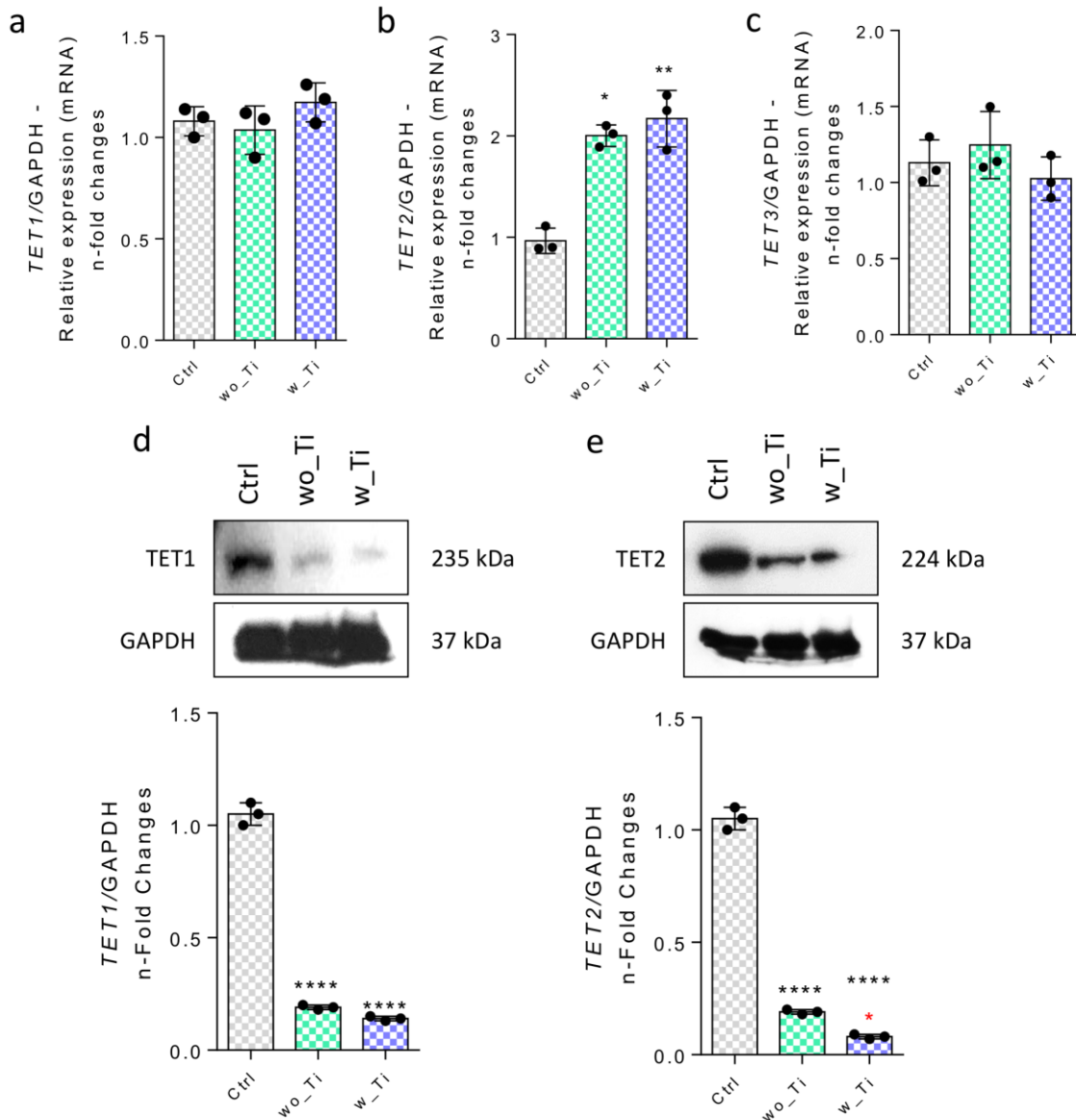


Fig.6. Effect of titanium-enriched medium on TETs. Firstly, the gene's activities were evaluated by qPCR, as follows: TET1 (**a**), TET2 (**b**), and TET3 (**c**), where GAPDH gene was used to normalize the data as a housekeeping gene. Thereafter, the protein profile was evaluated by western blot by resolving 75 μ g of sample per lane and later incubated with specific antibodies. A representative immunoblots of TET1 (**d**) and TET2 (**e**) are presented. Densitometric analysis of immunoblots was normalized to the protein ratio of

controls and GAPDH, here used as loading control (housekeeping control). Differences were considered statistically significant when $*p < 0.04$, $**p < 0.001$, $****p < 0.0001$ when compared with the control (Ctrl) group; and $*p < 0.04$ when compared to wo_Ti and w_Ti. The graphs bring mean $\pm$ standard deviation of three independent experiments. Note: TET: TET methylcytosine dioxygenase; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; Ctrl: control group; wo_Ti: medium enriched with titanium without surface modification; w_Ti: medium enriched with titanium with surface modification. The experiment was performed in triplicate ($n=3$).

4. DISCUSSION

During the bone growth along metallic devices osteointegration process, well-controlled angiogenesis is expected by stimulating the endothelial cell viability and proliferation, supplying the newly formed bone with cells, nutrients, and metabolite changes. Previously, we have shown that titanium-based implants induce dynamic microenvironment changes by releasing titanium molecules [39], which are very active in bone cells [40]. Considering the heterogenicity and plasticity of cells surrounding implants, this is necessary to understand the behavior of endothelial cells in this complex biological scenario related to wound healing. As seminal works have strongly listed epigenetic marks in governing the capacity of cells to respond to environmental changes [41], we have addressed this issue in this study by investigating the requirement of enzymes able to control the (de)methylation of DNA strands as well as (de)acetylation of histones, which directly compromises the capacity of cells in activating specific genes in so response to the environment changes, as well as impacting their capacity to respond to biomaterials effects.

Endothelial cells compose not only a barrier in blood vessels but also play multiple roles in distinguished pathophysiological events, such as the control of vasculature tonus and capacity to interact with the blood and surrounding cells at the same time, contributing with major signaling able to drive angiogenesis [42]. To note, angiogenesis is an essential event expected during biomaterial interaction with the host tissues and cells and compromising the success or failure of dental implants during osseointegration biological steps. We have over the last years stated that titanium-based dental implants modulate cells away thought of specific signaling pathways requiring

most of the time the control of reactive oxygen species (ROS) [43]. Additionally, it is known that ROS drives the activity of relevant proteins intracellularly such as protein tyrosine phosphatases (PTPs), which PTP1B has distinguished control in osteoblast cell adhesion [43]. Although some progress has been gathered in this regard, the capacity of the titanium-based implants in modifying the microenvironment is barely known and it brings new perspectives to know the role of epigenetic machinery in cells responding to it. Among others, our data show a potential constitutive expression of SIRT1 in endothelial cells, and it might be required to modulate ROS, once ROS production was previously reported in response to titanium-enriched medium and oxidative stress compromises the vascular function of SIRT1 [44].

Additionally, the capacity of endothelial cells modulates the gene expression was also investigated here by assaying HDACs, DNMTs, and TETs. Firstly, considering the involvement of histone lysine acetylation balance, our data shows a likely pivotal role of HDAC 6 in endothelial cells responding to titanium and it is shown to spread along the cytoplasm [45,46]. This is time to correlate HDAC6 with cytoskeleton rearrangement and consider it during cell adhesion, migration, and proliferation. In fact, HDAC6 has been previously listed in contributing to cytoskeleton rearrangement of endothelial cells with important repercussion on angiogenesis [47]. Previously, we have extensively shown that titanium-enriched medium affects the molecular machinery related to cytoskeleton rearrangement upon integrin activation and in conjunction, these findings reveal a critical role for HDAC6 in mediating titanium-induced cellular events.

All of DNMT1, DNMT3A and, TET2 genes were higher in endothelial cells responding to titanium-enriched medium, but it doesn't necessary reflect on the protein pattern. Mechanistically, this discrepancy between gene activity and protein profile strongly suggest an efficient posttranscriptional machinery in endothelial cells such as microRNAs and long non coding RNA (lnc-RNA). Specifically, DNMT1 is a conserved methyltransferase necessary to maintain global methylation of DNA strands, while DNMT3A and DNMT3B are *de novo* produced methyltransferases [41,48,49]. On the other hand, TETs are involved in an active DNA demethylation pathway by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [50–52] and they are important to demethylate DNA of cells responding to environmental changes, as titanium or other metallic biomaterials used in medicine or dentistry. Additionally, this might be explored in predictable tools as bioreactors responding to implantable biomaterials [53–

56] once nowadays cultures of blood vessels in bioreactors have been reported [57,58]. Also, to know these effects of the titanium in cells and humans within field is relevant also in considering novel propositions in bioengineering research and, as these enzymes are druggable, they are target to specific design of small molecules able to modulate their activities looking for enhancing the angiogenesis and it might accelerate the recovering of patients by improving the osteogenesis surrounding the titanium-based implants.

5. CONCLUSION

Taking into account, these findings support the hypothesis that titanium promotes a surrounding microenvironment changes and it repercusses on the capacity of endothelial cells responds to microenvironment by modulating epigenetic-players, where HDAC6 seems developing a crucial role. In perspective, it opens new way to better evaluate whether HDAC6 modulates endothelial cell cytoskeleton rearrangement during angiogenesis during titanium osseointegration.

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CAPITULO 3

Angiocrine molecules sourced from mechanotransduction-related endothelial cell signaling explain the successful outcome of titanium in osseointegration

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Keywords: Titanium; dental implants; osseointegration; paracrine signaling; osteogenesis; endothelial cells; mechanosignaling.

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ABSTRACT

To investigate whether this crosstalk was happening in this context, we have considered also the mechanotransduction-related endothelial cell signaling underlying laminar shear stress (up to 3 days) and this angiocrine factors-enriched medium was harvested further to use exposing pre-osteoblasts (pOb) up to 7 days in vitro. Two titanium-surfaces were considered as follows: double acid etching treatment (w_DAE) and machined surfaces (wo_DAE). These surfaces were used to conditionate the cell culture medium as recommended by ISO10993-5:2016 and this titanium-enriched medium as latter used to expose ECs. Firstly, our data shows there is a difference between them in releasing Ti molecules to the medium, providing a very dynamic surfaces, where the w_DAE releases around 25% higher (4ng/mL), when in comparison to wo_DAE (3 ng/mL). Importantly, the ECs uptake few amounts of this titanium content up to 3 days in culture. However, when this conditioned medium considering the angiocrine factors released from ECs was used to expose pOb up to 7 days, the concentration of Ti was lesser than previously reported, it reaching around 1 ng/mL and 2 ng/mL, respectively. Thereafter, pOb exposed to this angiocrine factors-enriched medium present a significant difference if also considering the mechanosignaling subjected the ECs. Shear-stressed ECs show an adequate crosstalk with osteoblasts, stimulating the higher expression of Runx2 gene driving the higher expressions of Alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin. Mechanotransduction-related endothelial cell signaling as a source of angiocrine molecules also stimulates the higher expression of Col3A1 gene in osteoblasts, which suggest relevant protagonist during the trabecular bone growth. In fact, we have investigated the ECM remodeling by firstly evaluating the expression of genes related with it, and our data shows higher expression of matrix metalloproteinase (MMP) 2 and MMP9 in response to mechanosignaling-based angiocrine molecules, independently if considering w_DAE or the wo_DAE, and this profile reflects on the MMP2 and MMP9 activities, evaluated by gelatin-based zymography. Complimentarily, the ECM remodeling seems being a very regulated mechanism in mature osteoblasts during the mineralization process, once both TIMP metalloproteinase inhibitor 1 and 2 (TIMP1 and TIMP2, respectively) genes are significantly higher in response also to mechanotransduction-related endothelial cell signaling as source of angiocrine molecules. Altogether, our data shows the relevance of mechanosignaling in favoring ECs release bioactive factors periimplant, which are responsible to create a osteogenic microenvironment able to drive osteoblasts differentiation and modulating ECM remodeling. Taking it into account, it seems mechanotransduction-based angiocrine molecules explain the successful use of titanium during osseointegration.

Keywords: Osteoblasts; Endothelial Cells; Titanium; Angiogenesis; Mechanotransduction; Osteogenesis; Osseointegration.

1. INTRODUCTION

Over the last years, the bioactivity of dental implants has been searched along the materials development and a number of studies has gathered mechanical and biological properties expected to develop by novel materials in bone [1,2]. In this way, this is shown the relevance of the biological properties of implant's surfaces in mimicking natural tissues as bone, and on this end, several bioactive surfaces have been proposed by creating a nanostructured coating using mimetically molecules, such as hydroxyapatite, or only modifying the topography of implants by subjecting them to acid treatment to ameliorate the roughness of the surface [1,3–6]. Both strategies are very welcome during implants interacting with host tissues, presenting good outcomes in dental rehabilitation also considering comorbidities [7,8]. Altogether, these statements gather important value considering the life expectancy worldwide and the increased number of total and partial edentulism. Among the implantable devices, titanium and its derivate alloys are the mostly applied in both medical and dental surgeries to rehabilitee the patients considering its properties in better associating with bone cells and molecules upon osseointegration process [5,9].

Although titanium is used so far, very few is known about the relationship among bone cells, endothelial cells (ECs) and matrices along the osteogenesis at the periimplant microenvironment. In this scenario, it is expected the healing process requires the activity of bone cells in cross-talking with ECs driving the morphogenetic changes in housing cells during osteogenesis. Thus, the connection between host bone tissue and the quality of the implant's surface is crucial to mimic an ideal environment for healing [9–11]. Actually, osteoprogenitor cells are protagonists of this osseointegration mechanism and very few value is credited to ECs and angiogenesis. However, the effect of housing ECs along the periimplant microenvironment needs to be better evaluated in order to comprehend the harmony among cells and molecules. Angiogenesis deserves being considered not only as a conduit for delivering cells and molecules, but also playing active paracrine signaling tissue during wound healing by recapitulating the earlier proposed interactions between them during bone growth [12]. We considered the bioinspiration on embryogenesis stages must be better applied during biomaterial development looking for more biomimetically materials, where the surface of biomaterials can initiate important biological changes [13–16]. In most cases, cellular phenotype and quality of osseointegration are time-dependent and consequently have to monitor the cellular

response through biological players that control the intracellular pathways in response to biomaterial surface [16–20]. In this scenario, the capacity of the biomaterial in adsorbing proteins on their surface to favor later cells adhesion is a desirable properties for novel materials [21,22].

It is known that the cell adhesion process can occur in substrates and components of the extracellular matrix (ECM) upon extracellular integrins domains, which modify its intracellular portion and creates points of interaction with intracellular proteins, such as Focal Adhesion Kinases (FAK) and Src [22–28]. This supramolecular platform promotes an adaptation in the cytoskeleton structures and establishes important parameters regarding the survival of cells such as adhesion, proliferation, migration and differentiation [29,30]. Considering Focal adhesion kinase (FAK) and Src, the phosphorylation cascade is modulated by the capacity of those cells in guiding the ECM components breakdown by specific matrix metalloproteinases and cathepsins activities culminating immediately to the rearrangement of the cytoskeleton [9,11,31,32]. This mechanism related with ECM remodeling is expected along the implanted implants shelf-life and so, the surface of titanium continuously releasing signals able to activate cells to promote osteogenesis [32–34]. To the desirable osseointegration occurs, biocompatibility and osteo-inductivity processes will reflect in close interactions between the cytoskeleton and biomaterial surface [35] during the bone remodeling periimplant. On this sense, we have previously shown that titanium and its alloys are very dynamic materials and provide an important communication with surrounding tissues. Importantly, this mechanism related with the dynamism of titanium involves the modulation of enzymes able to control the phosphorylation balance intracellularly, such as the lower phosphorylation of PTP1B favoring the activity of FAK during osteoblast adhesion on dental titanium implants surfaces [36]. However, all of those findings open new perspective to better mimic the periimplant microenvironment now considering the involvement of ECs on this context, and we have addressed here the hypothesis that EC interfere on the activity of osteoblast during response to titanium in preliminary aspects of osseointegration.

Here, we investigated the influence of titanium-enriched medium in the metabolism of ECs and thereafter its effect on osteoblast was also considered. This is expected to gain on the proposing novel in vitro strategies as alternative methodologies able to minimize the use of experimentation-related animals, where angiocrine-released factors from EC responding to titanium can be used to expose pre-osteoblast (pOb). Considering a very similar strategy we have better addressed the effect of Cobalt-

Chromium on this regard and it was validate [37]. Furthermore, it is considered the effect of a angiocrine factors in a paracrine signaling dependent osteogenesis required during osseointegration of dental implants. Technically, we have also considered culturing the ECs responding to laminar shear stress looking for mimicking the tensional forces of blood flow, respecting the physiological mechanotransduction-related endothelial cell signaling.

2. MATERIAL AND METHODS

2.1. Materials and titanium alloys.

Titanium alloy discs were obtained from S.I.N. (São Paulo, SP, Brazil). Cell culture staffs were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland), and the cell culture media for cell experiments purchased from Nutricell Co (Campinas, São Paulo, Brazil). For qPCR technology, primers were obtained from Exxtend Oligo Solutions (Paulinia, São Paulo, Brazil), GoTaq® qPCR Master Mix (A6002) was obtained from PROMEGA (Madison, Wisconsin, USA). All analytical grade chemicals were purchased from Sigma – Aldrich (St. Louis, MO, USA).

2.2 Cell culture.

HUVECs (Human Umbilical Vein Endothelial Cells; also named ECs) and pre-osteoblasts (MC3T3-E1, subclone 4; pOb) were purchased from American Type Culture Collection-ATCC (Rockville, Maryland, USA; passage <20), and certified as mycoplasma-free cells. Routinely, the lineages were incubated at 37°C and 95% humidity, in an environment containing 5% of CO₂. The specific cells were cultured respecting the manufacturer recommendations and supplemented the cell culture medium with 1% of antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) and 10% Fetal Serum Bovine (FBS) (Nutricell, Campinas, SP, Brazil). The experimental workflow considering the response of osteoblasts to angiocrine factors and titanium was as follows: 1) Control (Ctrl) – pOb cultures maintained in a traditional supplemented α -MEM medium and O.M. (Osteogenic Medium), where osteoblasts were maintained under known osteogenic medium stimulant (osteoblast differentiation positive control); 2) Osteoblasts exposed to conditioned medium by ECs (both shear-stressed or not), here also considered a reference group to analyzing the effect of titanium; 3) pObs exposed to conditioned medium from

non-shear stressed ECs responding to titanium. The **Fig.1** (items 1-4, top of the panel) summarizes the experimental workflow used in this study. Different titanium modified surfaces were investigated: w_DAE: double acid-etched (w_DAE) or wo_DAE: without DAE.

2.3 Preparation of angiocrine factors-obtaining medium.

Initially, titanium discs were incubated within a Falcon® Conical Centrifuge Tubes (Glendale, Arizona, USA) containing FBS-free cell culture medium for 24 h, as recommended by ISO 10993:2016, when the samples were properly centrifuged to avoid any debris and later used to evaluate the behavior of cells (**Fig.1**). It was here expected that the surface named DAE should release titanium molecules. Furthermore, ECs were exposed to that titanium-enriched medium during 3 days, when the EC-conditioned medium containing angiocrine factors samples were duly collected to allow the titanium content. Important considering here that ECs were subjected shear stress as a testing group, as well as considering non-stressed cells as a comparable and reference group, avoiding the mechanical factors described to mimic hemodynamic forces of physiological blood flow (**Fig.1**). Both conditioned medium by ECs were considered in this study containing angiocrine factors, soluble molecules released by ECs from its metabolism in response to titanium soluble. Finally, the angiocrine factors containing medium was later used to expose osteoblasts during 7 days, when the cell samples were harvested to allow the molecular analysis.

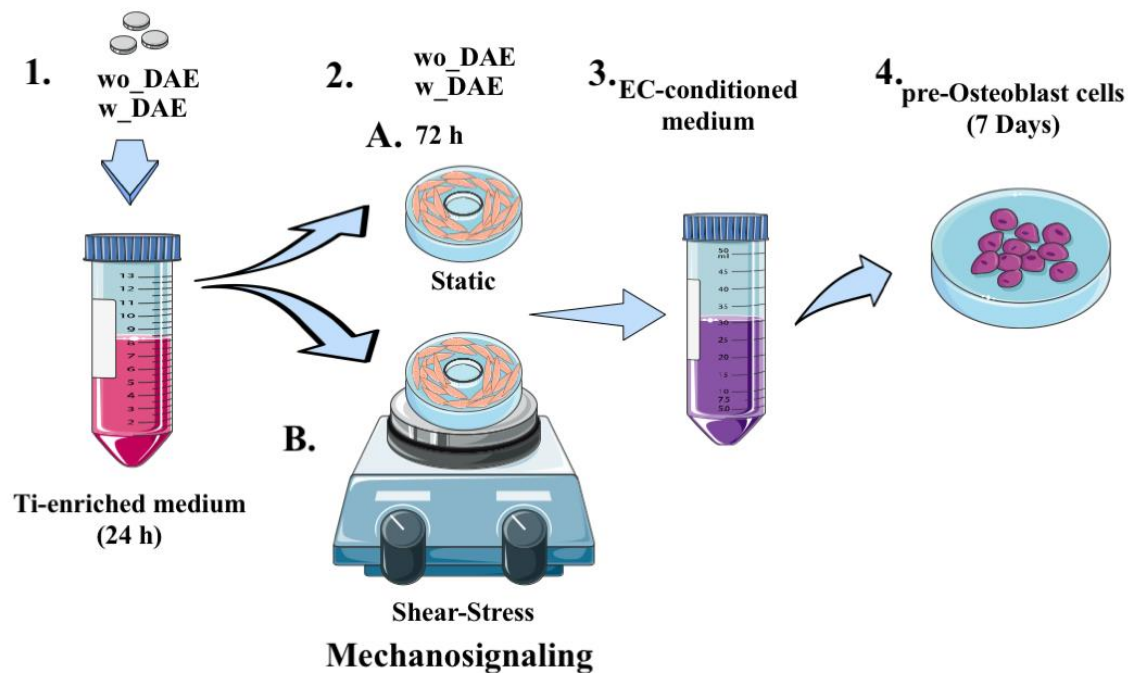


Fig.1. Schematization of the experimental flow and timeline. In order to better know about the relevance of endothelial cell (EC) on osteoblasts responding to titanium-enriched medium, a new model was proposed by incubating the body-of-proof (discs) of titanium subjected to two different surfaces: presenting DAE surface (*w_DAE*) and machined surface (*wo_DAE*). The discs were maintained into cell culture medium in conical tubes (1) up to 24 h in cell culture incubator. Thereafter, the Ti-enriched medium was used to expose ECs up to 3 days, respecting 2 experimental conditions: static, where ECs were maintained in conventional cell culture conditions, and dynamic, where ECs were maintained considering shear-stress. In both conditions, the EC cultures were exposed to Ti-enriched medium (2). The EC-conditioned medium was harvested after 3 days (3), centrifuged and later used to expose pre-osteoblasts (pOb) up to 7 days (4). Both cell culture medium and pObs were harvested separately to later evaluate MMP's activity and transcriptional profile of specific genes, respectively. Servier Medical Art models, licensed under a Creative Commons Attribution 3 License. Not ported; <https://smart.servier.com>.

2.4 Laminar shear-stress model was applied to mimic mechanotransduction-related endothelial cell signaling related to blood flow.

Modified cell culture dishes were previously prepared by medical silicone bonding smaller 60mm diameter cell culture dishes at the center of 100mm diameter. This modified cell culture dishes were later sterilized by a UV light up to 30 min prior to use

along the experimental workflow and timeline. Important to mention that the any cytotoxicity was detected by cells exposing to medical silicone (data not shown). Semi-confluent (around 85%) monolayers of ECs were cultured in the peripheral ring of the modified dishes area to simulate blood flow mimicking the laminar tensional forces obtained from an orbital *shear-stress* modulus as previously reported (Abe and Berk 2014; de la Paz et al. 2012; Pinto et al. 2019; R. A. da Silva et al. 2019). All experiments were conducted by an orbital shaker (Scilogex, Rocky Hill, CT, USA) into CO₂ incubator at 37°C and 5% CO₂ atmosphere. The calculation of shear stress was described earlier (Pinto et al. 2019).

2.5 Titanium content was quantified by GFAAS.

To titanium quantification (Ti), 20 µL of culture medium content was further to use for the analysis as depicted in the **Fig.1**. The conditioned culture medium was used to expose ECs and aliquots were collected to evaluate. Next, the Ti-enriched medium was later used to expose ECs up to 3 days, and thereafter the medium carries the angiocrine factors to osteoblasts up to 7 days. Each stage was saved aliquots to measure the titanium content in order to infer some conclusion about the potential uptake by the cells. All samples were measured in the GFAAS technology using a SHIMADZU model AA-6800 atomic absorption spectrometer. Such a spectrometer has a background absorption module attached in a self-reverse system (SR) with a pyrolytic graphite tube integrated platform and an ASC-6100 automatic sampler. A Shimadzu hollow cathode lamp of Ti operated at a minimum current of 12 mA and a maximum current of 600 mA (currently used in background correction – BG). Argon was used as the inert gas, and a constant flow of 1 L min⁻¹ was maintained during the entire heating program except for the atomization stage, during which the gas flow was stopped. The absorbance measurements (based on the peak area) were carried out in triplicate. The graphite tube's heating program optimized for titanium determination was based on the procedure described by Silva et al. [42] with some modifications and it is described in following: Drying temperature – 120/250°C; Pyrolysis temperature – 900°C; Atomization temperature – 900°C and Cleanup temperature – 2600°C.

2.6 mRNA isolation and qPCR analysis

Osteoblasts cultures responding to different condition detailed earlier here were properly collected using trizol method, exactly as recommended by the manufacturer (Life Sciences, Thermo Fisher Scientific Inc., Waltham, MA, USA, when the total RNA was carried out by adding DNase I (Invitrogen, Carlsbad, CA, USA) and the measuring performed in a Biotek microplate reader (Synergy multi-mode reader, Biotek, USA). cDNA was synthesized by performing High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed evaluating the expression of the genes which are listed in **Table 1**, in a total of 10 μ L, containing PowerUp™ SYBR™ Green Master Mix 2x (5 μ L; Applied Biosystems, Foster City, CA, USA), 0,4 μ M of each primer, 50 ng of cDNA and nuclease-free water. The graphs bring the relative amounts of the transcripts using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, using the cycle threshold (Ct) method.

Table 1. Genes, primers sequences and running condition.

Gene	Primer	5'-3' Sequence	Work Conditions
Runx2	Forward	GGACGAGGCAAGAGTTTCA	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	TGGTGCAGAGTTCAGGGAG	
Osterix	Forward	CCCTTCCCTCACTCATTTCC	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	CAACCGCCTTGGGCTTAT	
ALP	Forward	GAAGTCCGTGGGCATCGT	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	CAGTGCGGTTCCAGACATAG	
Osteocalcin	Forward	AGACTCCGGCGCTACCTT	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	CTCGTCACAAGCAGGGTTAAG	
BSP	Forward	GTACCGGCCACGCTACTTTCT	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	GTTGACCGCCAGCTCGTTTT	
Col1a1	Forward	ATGACGTGATCTGTGACGAGAC	

	Reverse	TTCTTGGTCGGTGGGTGAC	95°C - 15s; 60°C - 30s; 72°C - 60s
Col3a1	Forward	GACCTGAAATTCTGCCATCC	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	GCATGTTTCCCCAGTTTCC	
Mmp2	Forward	AACTTTGAGAAGGATGGCAAGT	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	TGCCACCCATGGTAAACAA	
Mmp9	Forward	TGTGCCCTGGAACACACGAC	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	ACGTCGTCCACCTGGTTCACCT	
Timp1	Forward	ATCCTCTTGTTGCTATCACTG	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	GGTCTCGTTGATTTCTGGG	
Timp2	Forward	GCAACAGGCGTTTTGCAATG	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	CGGAATCCACCTCCTTCTCG	
Gapdh	Forward	AGGCCGGTGCTGAGTATGTC	95°C - 15s; 60°C - 30s; 72°C - 60s
	Reverse	TGCCTGCTTC ACCACCTTCT	

2.7 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX).

Osteoblasts and ECs were cultured directly on the titanium based surfaces, when the morphological changes were monitored up to 3 days. SEM images were acquired and EDX analysis were performed subjecting the samples to scanning electron microscopy by FEI (Quanta 200; Hillsboro, Oregon, EUA), attached to the secondary electrons detector. Individual elements composition of materials (carbon and titanium) was analyzed by Energy Dispersive X-ray (EDX) module using a proper EDX detector from Oxford INCA 250p20. All collected data has a semi-quantitative profile by evaluating 3 different areas of the same sample which was correlate between the 3 measurements average at 20 kV energy operation. Field-of-view was defined by 500 times of magnification.

2.8 Gelatin proteolysis-based zymography

During the experimental model, aliquots of the medium conditioned by ECs or osteoblasts were harvested to further estimate the activity of matrix metalloproteinases (MMP) using a widely explored gelatin proteolysis-based zymography. In order to avoid the debris, the samples were properly centrifuged by applying 14,000 rpm up to 15 min and the protein amount was assayed by Lowry protocol [43]. The previously quantified protein was later determinant to guide the load of the pool of the proteins resolved using a 12% polyacrylamide gel containing 4% gelatin, the renaturation of proteins was carried by using Triton X-100 aqueous solution (2% w/v), up to 18 hours in proteolysis buffer (Tris – CaCl₂) at 37°C and then stained with Coomassie Blue R- 250 dye solution 0.05% up to 3 h. The stained gels were washed in 30% methanol (v/v) and 10% glacial acetic acid solution (v/v) containing solution. The bands were considered to measure the gelatinolytic activity of metalloproteinases 2 (MMP2, ~62 kDa) and 9 (MMP9, ~84 kDa), and then they were subjected to the densitometric analysis using software ImageJ (Bethesda, MD, USA) [44].

2.9 Statistical analysis

The replicates of the each experiment was represented as mean $\pm$ standard error deviation (SE) (n = 3). Significant differences were when $p < 0.05$. The statistical data were collected using analysis of variance (two-way ANOVA) with multiple comparisons of all pairs of groups applying Bonferroni's correction posttest, or nonparametric analysis. Further, the significance level was considered when $\alpha = 0.05$. All data were obtained using GraphPad Prism 7 program (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

Dynamic interaction of titanium surface with the microenvironment

We have investigated the morphology of ECs and osteoblasts directly interacting on the both wo_DAE and w_DAE surfaces up to 3 days. SEM images show expected morphological changes of both cells (**Fig.2a**). Additionally, these samples were subjected to EDX analysis, when it was possible to map the distribution of carbon and titanium spread on the evaluated surfaces (**Fig.2a**). Thereafter, to determine whether it was significant the release of titanium from the titanium-based alloys, we have used GFAAS technology. Briefly, the discs were maintained into conical tube in FBS-free cell culture

medium during 24 h at 37°C, when the titanium-enriched medium was harvested and subjected to analysis. Our data shows that w_DAE (~4ng/mL) releases higher amount of titanium than wo_DAE (~3ng/mL) (**Fig.2b**), and this difference might be in relation with the roughness promoted by DAE. Thereafter, this medium was further used to expose ECs, and the **Fig.2c** brings clear evidences that there is no significant uptake of this released titanium by ECs up to 3 days of exposition, once the amount of titanium soluble found in the cell culture medium was very similar that shown earlier the exposition, it being ~4ng/mL for w_DAE, and ~3ng/mL for wo_DAE. Conversely, the osteoblast have uptake ~2ng/mL up to 7 days of exposition, independently if w_DAE or wo_DAE (**Fig.2d**).

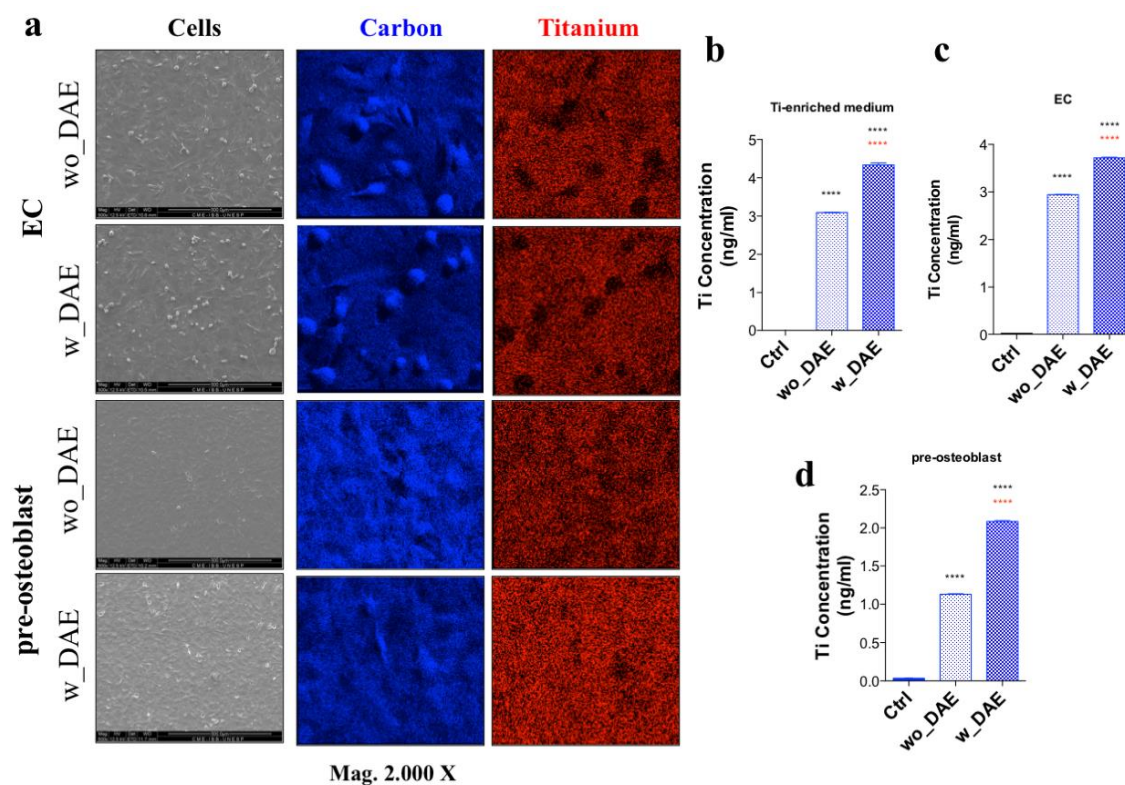


Fig.2. Titanium surfaces dynamically interact with the microenvironment. Cells were cultured directly on the both titanium based surfaces wo_DAE and w_DAE up to 3 days and technically prepared to SEM analysis (**a**). Additionally, carbon and titanium elements distribution on the surfaces were also investigated using EDX analysis. Also, the released titanium was monitored in the conditioned medium, it being around 3 ng/ml and 4 ng/ml from wo_DAE and w_DAE, respectively (**b**). This Ti-enriched medium was after used to expose ECs up to 3 days, when a new aliquot was measured considering the

The periimplant microenvironment requires a synchrony of events (involving molecular and cellular issues), able to drive osteogenesis and consequently accelerating the osseointegration of implants. Although titanium has been widely used in biomedical application, the molecular and cellular crosstalk governing its biocompatibility in bone needs to be better addressed. Here, we have now investigated whether ECs impacts the titanium effect on osseointegration process by paracrine modulating osteoblast's phenotype. Firstly on this sense, it was shown that angiocrine soluble factors-containing medium was able to activate classical transcription factors of early osteogenic phenotype mechanism. Importantly, it is shown here the relevance of mechanotransduction-related endothelial cell signaling in releasing those angiocrine factors able to reach significance regarding both Runx2 and Osterix genes. Runx2 is a master switch for inducing osteoblast differentiation. Additionally, this response was even more significant considering the mechanotransduction-related endothelial cell signaling responding to wo_DAE (Fig.3a,b).

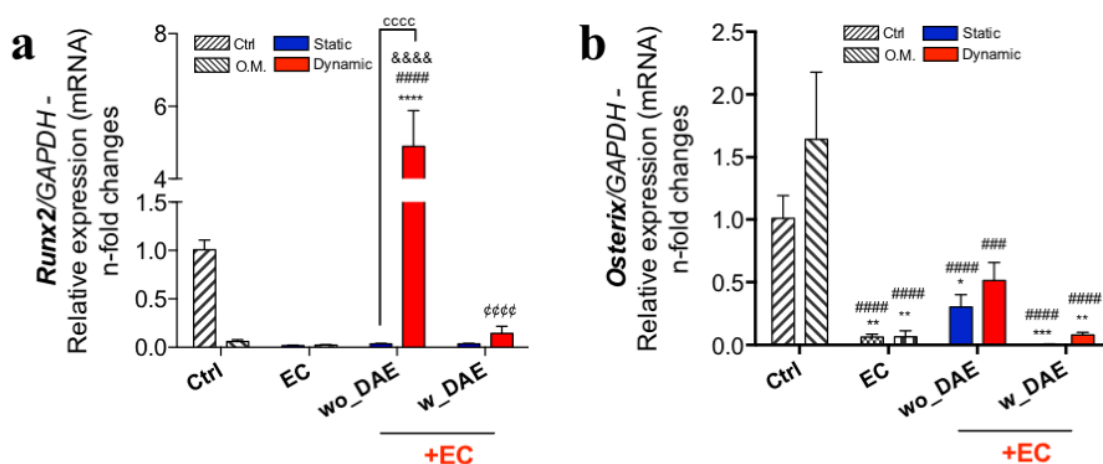


Fig.3. Osteogenic transcription factors Runx2 and Osterix genes are modulated by angiocrine factors. We have evaluated whether angiocrine factors from ECs participate on the osteoblast response to titanium-enriched medium. Osteoblasts samples were harvested and later used to evaluate the activation of transcription factors Runx2 (**a**) and Osterix (**b**) genes. Both of genes were higher in osteoblasts exposed to angiocrine factor sourced from mechanotransduction-related endothelial cell signaling, independently if wo_DAE or w_DAE. However, regarding Runx2 it seems wo_DAE developing a better performance. The experimental workflow brings the analysis of different control cultures, as follows: Ctrl: cultures were maintained under conventional condition; O.M.: cultures were under osteogenic medium condition; EC: osteoblasts were exposed to angiocrine factors obtained by EC under shear stress (dynamic) or non-subjected to SS (static). The qPCR running data of specific genes was later normalized by using GAPDH as a housekeeping gene. Statistics were considered significant, when: * $p < 0.0223$; ** $p < 0.0014$; *** $p < 0.0007$; p**** $p < 0.0001$; ### $p < 0.0008$; #### $p < 0.0001$; &&&& $p < 0.0001$; cccc $p < 0.0001$; and cccc $p < 0.0001$.

Thereafter, until considering the osteogenic phenotype, genes related with final events of osteoblasts differentiation were also investigated in response to titanium-conditioned medium in association with angiocrine factors, mature osteoblasts express alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN). Regarding ALP gene activation, our data clearly shows there is an influence of ECs on osteoblast responding to titanium-enriched medium. Importantly, both surfaces promotes higher ALP expression when titanium was associated with angiocrine factors released by shear-stressed ECs and strongly suggesting the mechanotransduction-related endothelial cell signaling in supporting ALP gene expression in osteoblasts. Here, there was significant difference considering the both evaluate surfaces: in response to wo_DAE, ALP gene expression was ~35-fold changes higher, while w_DAE promoted surprisingly ~150-fold changes higher when compared to the control (Ctrl) (**Fig.4a**). Importantly, O.M. promotes higher expression of ALP gene, and it can be considered on validation regard of this biological model. Additionally, the ALP expression profile shown here was very similar that found considering osteocalcin gene expression (**Fig.4c**). Specific to osteocalcin, shear-stressed EC-obtained angiocrine factors (without association with titanium) was sufficient to promote higher expression of osteocalcin.

Bone sialoprotein (BSP) gene was also investigated in this context, and it was very similar profile found regarding Runx2 gene behavior. Mechanotransduction-related endothelial cell signaling seems contributes actively as source of angiocrine factors, necessary to preserve an osteogenic microenvironment mainly considering in association with titanium-enriched medium by wo_DAE (**Fig.4b**)

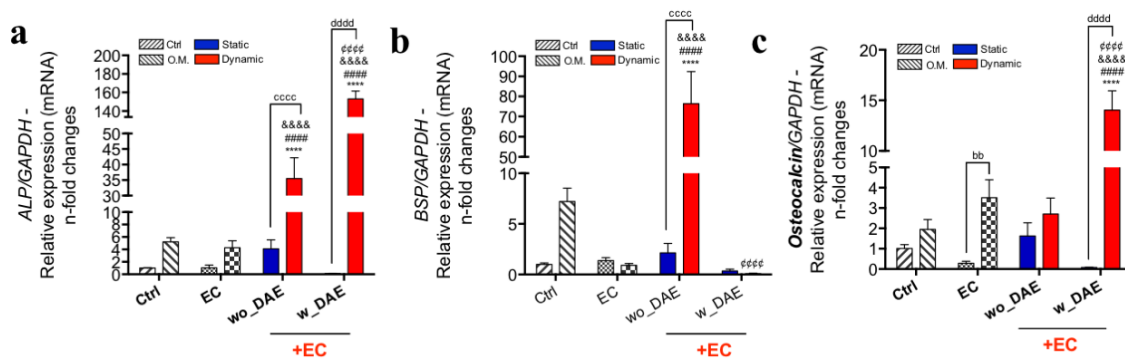


Fig.4. Differentiated osteoblast gene markers were higher in response to angiocrine factors obtained from EC. Again, the hypothesis of angiocrine factors contributing with the titanium response looking for osteogenic phenotype of osteoblasts was until investigated considering the expression of genes related with late stages of osteoblast differentiation. Thus, alkaline phosphatase (ALP; **a**), bone sialoprotein (BSP; **b**) and osteocalcin (**c**) genes were properly investigated in this way. Although presenting very similar response to mechanotransduction-related endothelial cell signaling, BSP seems also consider the effect of wo_DAE. The experimental workflow brings the analysis of different control cultures, as follows: Ctrl: cultures were maintained under conventional condition; O.M.: cultures were under osteogenic medium condition; EC: osteoblasts were exposed to angiocrine factors obtained by EC (endothelial cell) under shear stress (dynamic) or non-subjected to SS (static). The qPCR running data of specific genes was later normalized by using GAPDH as a housekeeping gene. Statistics were considered significant, when: $p^{****}<0.0001$; $####p<0.0001$; $****p<0.0001$; $****p<0.0001$; $bbp<0.0069$; $bbbbp<0.0001$; $ccccp<0.0001$; $ddddd<0.0001$.

3.2. Mechanotransduction-related endothelial cell signaling drives angiocrine factors able to require Col3A1 and profile of ECM remodeling in differentiating osteoblasts.

As osteoblasts are responsible for growing the new bone, also intermediating the synthesis of organic and inorganic portions of bone extracellular matrix (ECM), we have also investigated the expression of genes that are necessary and sufficient to induce mineralization of this ECM. Here, we have complementarily evaluated the capacity of angiocrine factors in association with titanium in trigger intracellular signaling in osteoblast able to drive the expression of Col1A1 (**Fig.5a**) and Col3A1 genes (**Fig.5b**). Considering Col1A1 gene, it was higher in O.M., as expected, and ~5-fold changes higher in angiocrine factor released in response to static condition of ECs responding to wo_DAE, suggesting it does not being dependent of mechanotransduction-related endothelial cell signaling. On this sense, the osteoblasts responding to EC also present higher expression of Col1A1 gene. Conversely, Col3A1 gene was dependent of mechanotransduction-related endothelial cell signaling obtaining angiocrine factors in response also to wo_DAE, it reaching ~2.5 fold changes higher than the control (Ctrl) (**Fig.5b**).

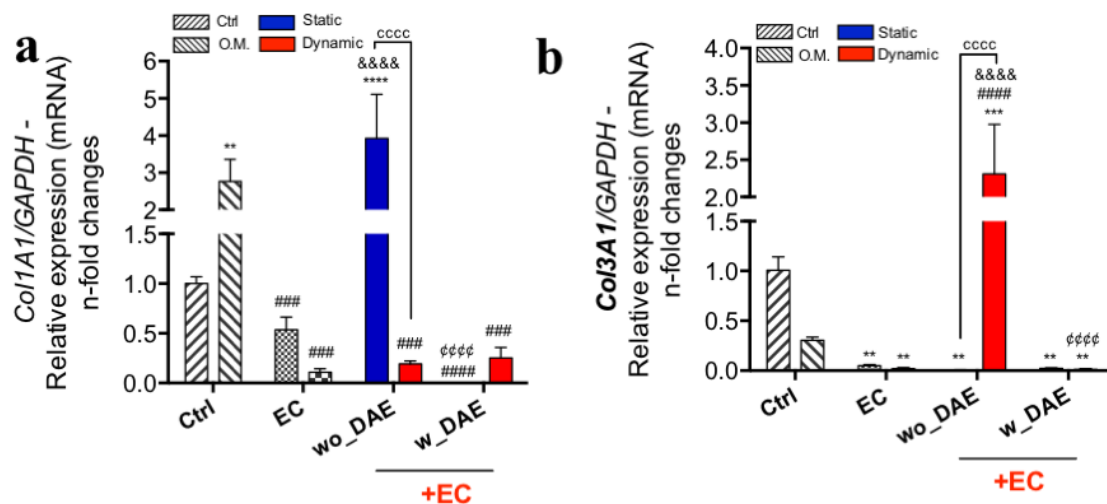


Fig.5. Col3A1 gene was higher in response to mechanotransduction-related endothelial cell signaling sourced angiocrine factors. Considering the bone ECM synthesis, in response to angiocrine factors, Col1A1 (**a**) and Col3A1 (**b**) genes were evaluated. Our data shows an enhanced expression of both genes in response to wo_DAE. The experimental workflow brings the analysis of different control cultures, as follows: Ctrl: cultures were maintained under conventional condition; O.M.: cultures were under

osteogenic medium condition; EC: osteoblasts were exposed to angiocrine factors obtained by EC (endothelial cell) under shear stress (dynamic) or non-subjected to SS (static). The qPCR running data of specific genes was later normalized by using GAPDH as a housekeeping gene. Statistics were considered significant, when: ** $p < 0.0086$; *** $p < 0.0005$; **** $p < 0.0001$; ### $p < 0.0008$; #### $p < 0.0001$; &&&& $p < 0.0001$; €€€€ $p < 0.0001$; and cccc $p < 0.0001$.

Also, the balance of expression of genes related with ECM remodeling was evaluated by checking the expression of MMPs and TIMPs (**Fig.6**). MMPs gene was significantly higher (~1.5 fold changes; **Fig.6**) in response to wo_DAE in conjunction of angiocrine factors obtained in shear stressed ECs, while conversely MMP9 gene developed different effect, here being higher in osteoblasts exposed to w_DAE in conjunction of shear-stressed ECs (**Fig.6b**). MMP2 gene activation seems reflect the profile of Col3A1 gene behavior. Additionally, both TIMP1 (**Fig.6c**) and TIMP2 (**Fig.6d**) genes were also higher in response to angiocrine factors from shear-stressed ECs, independently if exposed to wo_DAE or w_DAE. Finally, MMPs activities were also evaluated by gelatin proteolysis-based zymography (**Fig.7a**), and the both profile of those MMPs reflect directly the gene expression of both MMP2 (**Fig.7d**) and MMP9 (**Fig.7b,c**), with significant effect of ECs releasing angiocrine soluble factors in synergism with titanium-enriched medium.

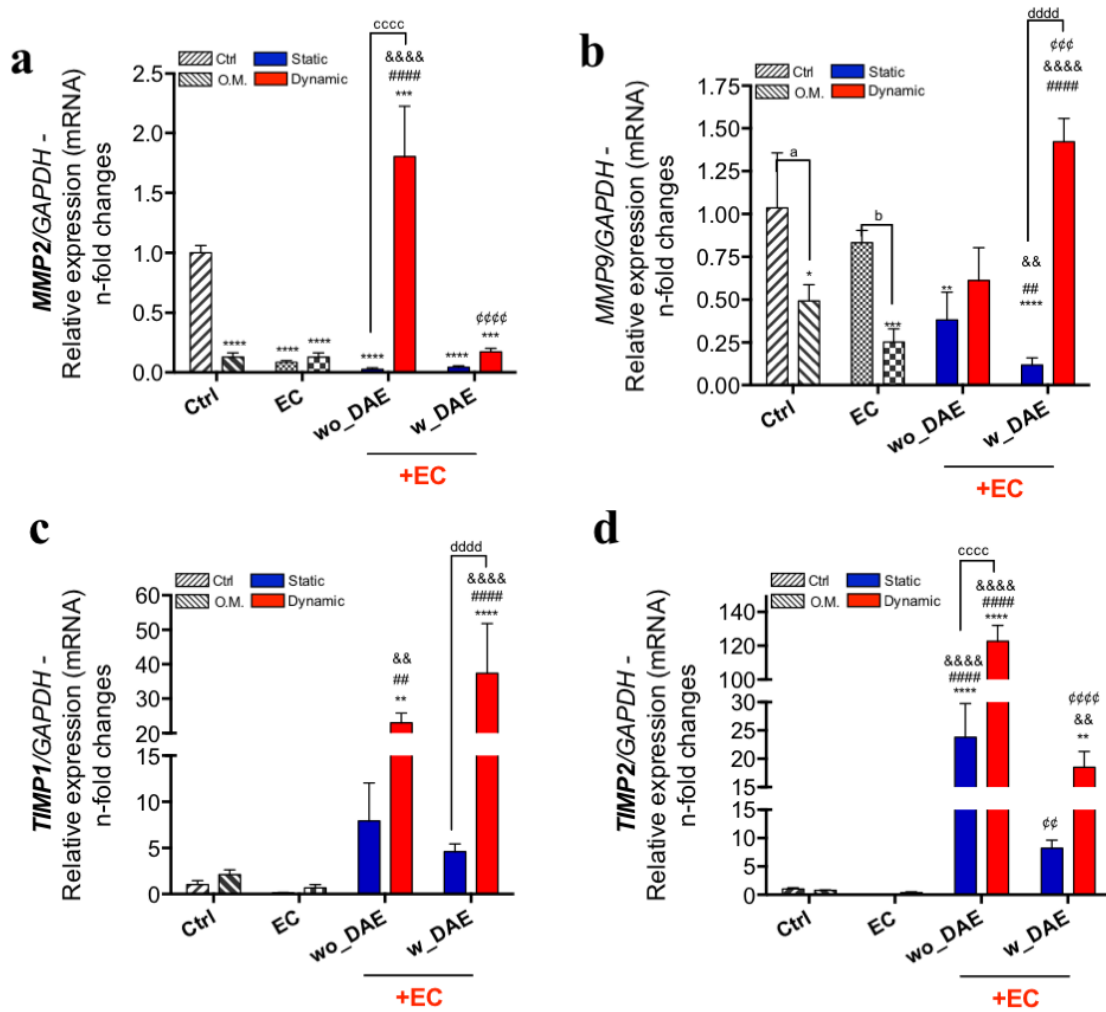


Fig.6. Bone ECM remodeling-related gene markers are finely modulated by mechanotransduction-related endothelial cell signaling sourced angiocrine soluble factors. Now, some advance was achieved considering the bone ECM remodeling by evaluating matrix metalloproteinases (MMP)2 (a) and MMP9 (b), as well as their tissue inhibitor of metalloproteinases 1 (TIMP-1) (c) and TIMP2 (d) genes were also investigated. The experimental workflow brings the analysis of different control cultures, as follows: Ctrl: cultures were maintained under conventional condition; O.M.: cultures were under osteogenic medium condition; EC: osteoblasts were exposed to angiocrine factors obtained by EC (endothelial cell) under shear stress (dynamic) or non-subjected to SS (static). The qPCR running data of specific genes was later normalized by using GAPDH as a housekeeping gene. Statistics were considered significant, when: * $p < 0.0223$; ** $p < 0.0086$; *** $p < 0.0005$; **** $p < 0.0001$; # $p < 0.0300$; ## $p < 0.0027$; ### $p < 0.0008$; #### $p < 0.0001$; & $p < 0.0343$; && $p < 0.0035$; &&& $p < 0.0009$; &&&& $p < 0.0001$;

^{cccc}p<0.0001; ^ap<0.0208; ^bp<0.0117; ^{bb}p<0.0034; ^{bbbb}p<0.0001; ^{cccc}p<0.0001;
^{dddd}p<0.0001.

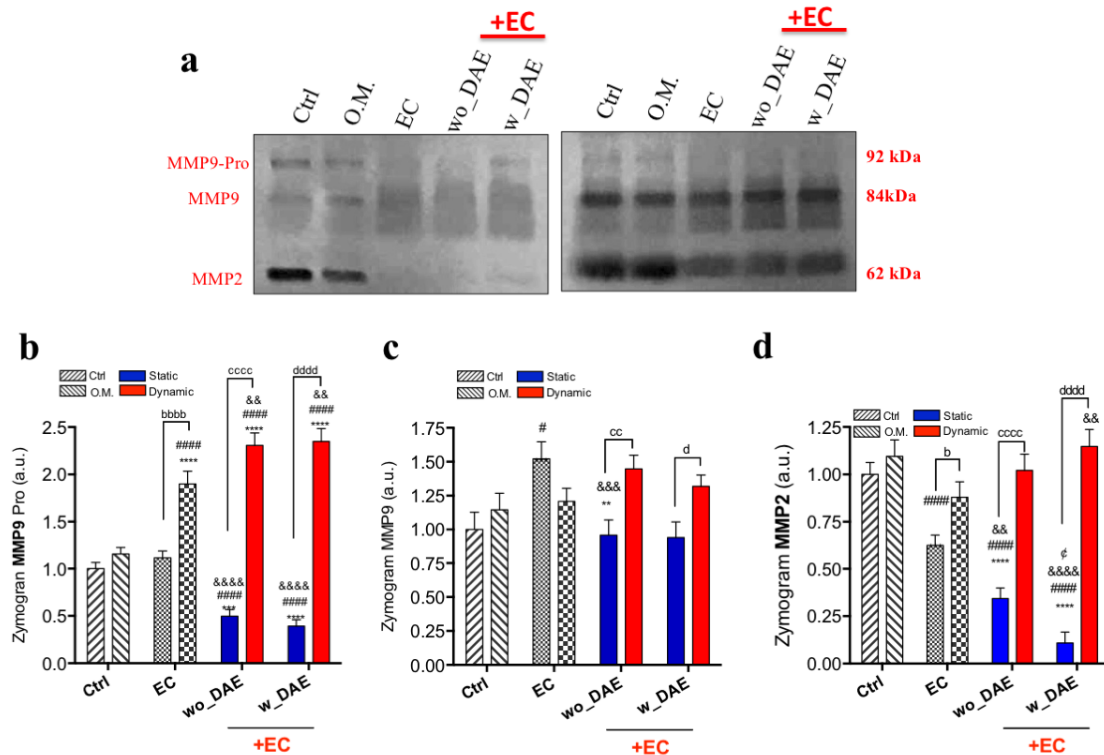


Fig.7. MMPs activities in Ob are modulated by angiocrine soluble factors sourced from mechanotransduction-related endothelial cell signaling. Complimentarily, MMP2 (**d**) and MMP9's (**b,c**) activities were investigated considering a gelatin proteolysis-based zymography, as it is displayed a representative zymogram (**a**). The experimental workflow brings the analysis of different control cultures, as follows: Ctrl: cultures were maintained under conventional condition; O.M.: cultures were under osteogenic medium condition; EC: osteoblasts were exposed to angiocrine factors obtained by EC (endothelial cell) under shear stress (dynamic) or non-subjected to SS (static). Statistics were considered significant, when: *p<0.0434; **p<0.0026; ***p<0.0006; ****p<0.0001; #p<0.0406; ##p<0.0091; ###p<0.0004; ####p<0.0001; &p<0.0065; &&p<0.0003; &&&p<0.0001; ^cp<0.0324; ^{cc}p<0.0011; ^ap<0.0482; ^{aaaa}p<0.0001; ^bp<0.0018; ^{bbbb}p<0.0001; ^{cc}p<0.0018; ^{cccc}p<0.0001; ^dp<0.0209; ^{dddd}p<0.0001

Finally, the **Fig.8** summarizes the main findings found in this study, where the protagonist of mechanotransduction-related endothelial cell signaling displays relevance on osteoblast differentiation along of its different stages and also compromising bone ECM remodeling. Altogether, gathering these data we have a draw of the osseointegration biology promoted by titanium alloys.

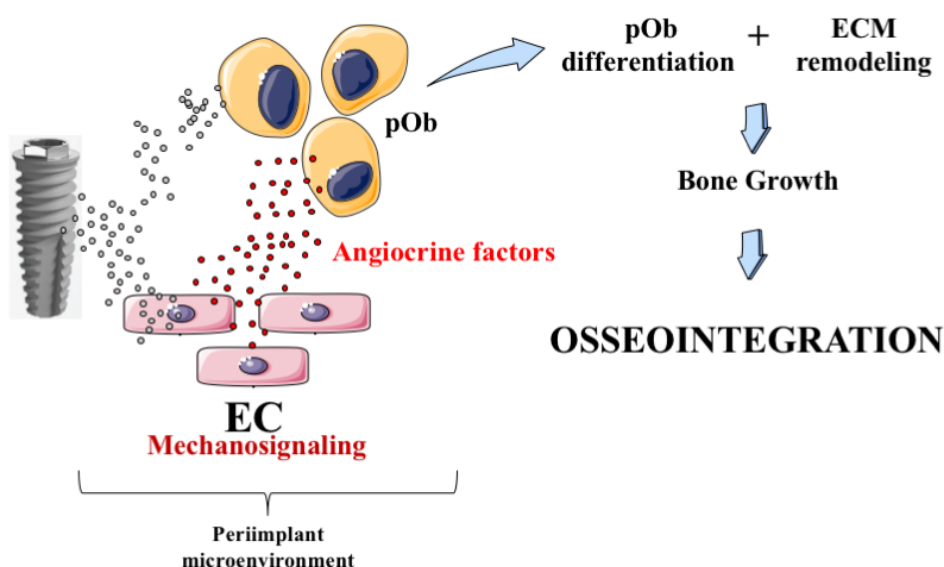


Fig.8. Schematization chart gathers the main findings of this study explaining the biocompatibility of titanium-based alloys. The released titanium from dental implants affect the biology of endothelial cells which release angiocrine factors under mechanotransduction-related endothelial cell signaling. This study shows this angiocrine factors in promoting morphogenetic changes in osteoblasts resulting on maturation of their differentiation mechanism with higher expression of osteogenic gene markers and their capacity in remodulating bone ECM. Altogether, these findings might explain the performance of titanium into bone, where the angiocrine factors sourced from mechanotransduction-related endothelial cell signaling is protagonist in this scenario.

4. DISCUSSION

Titanium has been so far the gold-standard material within biomaterial development field looking for predicting osseointegration. Although some progress has been achieved over the last years on this issue, there is still a lack of advance on comprehending the crosstalk among housing cells and molecules during the bone growth and tissue healing which might bring light on explaining the biology of osseointegration.

Over the last decade, we have proposed *in vitro* models to better understand the behavior of cells and molecules when interacting with biomaterials since ceramic to metallic devices and a map of molecules have been gathered to predict the better performance of cells in desirable materials and its surfaces [1,45–49]. In this study, we have addressed the gap on knowing the synchrony of cells and molecules during osseointegration by applying an *in vitro* biological model able to evaluate the potential communication between endothelial cells (ECs) and osteoblasts, mainly motivated by evaluating whether the pioneer findings by Kusumbe et al (2014) was recapitulated during the bone healing around the dental implants [12]. They have sufficiently shown that a specific EC physiologically drives the osteogenesis by assessing NOTCH signaling [50]. In this way, our data shows the relevance of laminar shear stress suffering ECs on guiding osteoblast differentiation responding to titanium released from dental implants. Furthermore, our data also suggests a protagonist for mechanotransduction-related endothelial cell signaling as source of bioactive molecules (angiocrine factors) able to coordinate the bone healing across the osseointegration mechanism.

Technically on this way, we have evaluated firstly the concentration of titanium released from dental implants up to 24 h and our data suggests a very dynamic microenvironment interacting actively with dental implants surfaces. This is time to mention that titanium-enriched medium was previously shown coordinating the activity of bone and endothelial cells [18,20,33,51,52]. Previously, we have shown the ability of this Ti-released amount in modulating the intracellular activity of protein tyrosine phosphatase 1B (PTP1B) and it results on the higher phosphorylation of Focal Adhesion Kinases (FAK) and it reinforces its capacity in interfering on osteoblast adhesion. This molecular draw depicts relevance to Reactive Oxygen Species (ROS) in oxidizing cysteines at the active site of PTPs and interfering on the phosphorylation balance in cells [36,53]. Now, our data brings a new discussion on this regard, once a slight amount of titanium was uptake by ECs and it might contribute on releasing angiocrine factors. However, it is clear that more needs to be found mainly considering the effect of ROS on the machinery able to express and release of those angiocrine molecules. Also, we intend that proteomic analysis is called for specifying and categorizing those molecules. Altogether, these angiocrine factors in conjunction to remaining titanium soluble in the conditioned medium impacting osteoblast differentiation thereafter uptaking around 2ng/mL up to 7 days in culture and it might prepare osteoblasts responding to angiocrine factors concomitantly. In fact, our data supports that shear-stressed ECs promotes an

adequate and bioactive microenvironment and titanium enhances significantly the expression of genes relate with osteoblast differentiation considering the full mechanism, since early stages by stimulating the expression of transcription factors such as Runx2 and Osterix to bone extracellular matrix remodeling favoring the activity of MMPs.

In agreement, our data supports this hypothesis when it was shown the higher expression of Runx2 gene (6-fold changes) in response to angiocrine factors in association with the responsiveness to titanium released molecules. Importantly, it is widely discussed that Runx2 starts the osteoblast differentiation along the early stages of this mechanism [54]. On this hand and now considering the late stages of osteoblast differentiation, we have shown a dependency of shear stressed ECs as source of angiocrine signaling in mature osteoblasts by up-modulating the expression of ALP, BSP and osteocalcin genes [55]. Altogether, this set of data validates the significance of mechanotransduction-related endothelial cell signaling in providing a bioactive and osteogenic microenvironment to support the maturation of osteoblasts and it is expected gains value thereafter permanent cell cycle withdrawal [56,57]. In details, the expression profile of ALP reached amount higher than 150-fold changes when osteoblasts were exposed to angiocrine factors in conjunction with titanium. At the same context, BSP profile reached approximately 80-fold changes. This physiological osteogenic condition acquired considering the synergism between angiocrine factors and titanium is unquestionable and might explain the performance of titanium during osseointegration mechanism.

Moreover, the bone growth and modeling requires a finely regulated bone ECM remodeling [11,56,58,59] . In order to better address this issue, we have firstly investigated whether this bioactive microenvironment might affect the expression of genes related with collagen synthesis. Our data shows that shear-stressed EC contributes with Col3A1 gene expression in osteoblast. Conversely, non-stressed EC modulates the expression of Col1A1 gene. Important, type III collagen has been correlated in regulating osteoblastogenesis and consequently impacts the quality and amount of trabecular bone [60]. It makes sense if considering the originality of the bone growing in contacting with dental implants independently if maxillary or mandible – the majority of bone interacting with implant surfaces is trabecular or bone marrow [61] . Additionally, if osseointegration is strongly influenced by the bone quality at the implant insertion site, titanium seems recapitulates the originality of the histology of bone tissue during the wound healing process at peri-implant microenvironment, and it distinguishes this mechanism as a

regeneration process, looking for avoiding implant's failure. More in this way, although playing a minimal role considering the primary stability, the trabecular bone certainly plays important role for implant bone healing and compromises its osseointegration.

This regenerative mechanism also requires the finely-modulated ECM remodeling and in this way, we have also investigated the ability of angiocrine factors in conjunction to titanium in modulating the expression and activities of MMPs. On this hand, our data shows clearly that both MMP2 and MMP9 genes were higher in osteoblasts responding to this bioactive microenvironment. Moreover, this profile of expression reflects on their activities of both MMPs. In addition, this bone ECM remodeling seems being finely modulated by the requirement of tissue matrix metalloproteinases inhibitors 1 (TIMP1) and TIMP2, once these genes presented a very similar profile of involvement. The physiological cues of ECM remodeling reveals a novel perspectives to tentative change the cell anchorage on the substrate and more insights are gathered to propose mechanotransduction-related endothelial cell signaling as protagonist of stages coordinating the crosstalk between EC and pOb during bone appositional growth at the periimplant environment.

5. CONCLUSION

Altogether, ours data gathers for the first time sufficient evidences to bring value on mechanotransduction-related endothelial cell signaling as prerequisite and source of angiocrine molecules which drives paracrine osteoblast differentiation and compromises osteogenesis during bone healing from behind the osseointegration mechanism of dental implants. Furthermore, when these findings are taken together can explains the success of titanium as metallic alloy in biomedical applications.

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DISCUSSÃO

Neste estudo, demonstramos que o meio condicionado pelo titânio desempenha um papel significativo no microambiente, levando a uma regulação importante dos mecanismos epigenéticos em células endoteliais. A epigenética envolve o estudo de mudanças hereditárias na expressão gênica que não envolvem alterações na sequência do DNA, mas permitem que as células respondam ao ambiente e modifiquem seu fenótipo (SCUMACI; ZHENG, 2023). Especificamente, as mudanças resultantes da maquinaria epigenética são mediadas por modificações químicas, como a acetilação de histonas (proteínas que auxiliam na compactação do DNA no núcleo) ou a metilação do DNA. A epigenética desempenha um papel crucial no desenvolvimento, diferenciação e resposta a estímulos ambientais das células (MANNA et al., 2023; SCUMACI; ZHENG, 2023). Neste estudo, demonstramos que ela pode ser modulada pelo meio condicionado pelo titânio, com diferenças notáveis entre as superfícies maquinadas e as superfícies tratadas com duplo ataque ácido.

Entre as moléculas epigeneticamente moduladas, o HDAC6 parece desempenhar um papel fundamental. Portanto, é pertinente avaliar se o HDAC6 influencia o rearranjo do citoesqueleto das células endoteliais durante a angiogênese durante a osseointegração de biomateriais (DA COSTA FERNANDES et al., 2021; FERNANDES et al., 2023). A HDAC6 (Histone Deacetylase 6) é uma enzima que regula a acetilação das histonas, mas também tem outros alvos, incluindo proteínas não histonas, como a tubulina. A HDAC6 desempenha um papel na remodelação do citoesqueleto, principalmente através da deacetilação da tubulina, o que pode afetar a estabilidade dos microtúbulos e o tráfego intracelular (SEIDEL et al., 2015). Portanto, avaliar se o HDAC6 está envolvido no processo de angiogênese durante a osseointegração do titânio é de grande interesse, pois pode fornecer informações sobre os mecanismos moleculares que regem a formação de novos vasos sanguíneos e a interação entre células endoteliais e o titânio liberado pelos implantes.

Essas investigações podem abrir novas perspectivas sobre os mecanismos moleculares subjacentes à angiogênese durante a osseointegração do titânio e podem ter implicações importantes para o desenvolvimento de estratégias terapêuticas destinadas a melhorar a formação de novos vasos sanguíneos, promovendo assim uma osseointegração mais eficaz e bem-sucedida de implantes dentários de titânio.

É importante destacar que essa capacidade de alterar a maquinaria epigenética das células endoteliais desencadeia uma sinalização parácrina importante com as células ósseas, contribuindo assim para a osteogênese (DA C. FERNANDES et al., 2021, 2023; FERNANDES et al., 2023; PATRICIA DA SILVA et al., 2023). Neste estudo, abordamos a lacuna no conhecimento sobre a sincronia entre células e moléculas durante a osseointegração, aplicando um modelo biológico in vitro capaz de avaliar a potencial comunicação entre células endoteliais (ECs) e osteoblastos. Essa abordagem foi motivada principalmente pela investigação de se os achados pioneiros de Kasumbe, Ramasamy e Adams (2014) podem ser recapitulados durante a cicatrização óssea ao redor dos implantes dentários (KUSUMBE; RAMASAMY; ADAMS, 2014).

Nesse sentido, nossos dados corroboram essa hipótese, mostrando um aumento na expressão do gene Runx2 (seis vezes maior em relação ao controle) em resposta aos fatores angiogênicos liberados pelas células endoteliais, como resultado das moléculas liberadas pelo titânio. Em conjunto, esses dados validam a importância da sinalização relacionada à mecanotransdução nas células endoteliais, fornecendo um microambiente bioativo e osteogênico que sustenta a maturação dos osteoblastos. Esse processo regenerativo também requer uma remodelação cuidadosamente regulada da matriz extracelular (TOKUHARA et al., 2019). Nesse sentido, também investigamos a capacidade dos fatores angiogênicos, juntamente com o titânio, de modular a expressão e as atividades das metaloproteinases da matriz extracelular (MMPs). Nossos dados demonstram claramente que os genes MMP2 e MMP9 foram expressos em maior quantidade nos osteoblastos em resposta a esse microambiente bioativo. Além disso, esse padrão de expressão também se refletiu nas atividades dessas MMPs. Além disso, a remodelação da matriz extracelular óssea parece ser cuidadosamente regulada pelos inibidores das metaloproteinases da matriz extracelular 1 (TIMP1) e TIMP2, uma vez que esses genes apresentaram um perfil de envolvimento muito semelhante.

Portanto, nossos resultados indicam que as alterações epigenéticas nas células endoteliais, em resposta ao meio condicionado pelo titânio que imita o microambiente perimplantar, são essenciais para a mudança fenotípica dessas células, o que, por sua vez, se reflete na diferenciação dos osteoblastos. Esse elo entre as células endoteliais e as células ósseas pode ser mediado por moléculas angiócrinas, definidas aqui como moléculas secretadas pelas células endoteliais que modulam, de forma parácrina, os efeitos em células diferentes, como os osteoblastos.

CONCLUSÃO

Em conjunto, nossos dados reúnem, pela primeira vez, evidências suficientes para atribuir valor à sinalização parácrina das células endoteliais, sob controle epigenético, como pré-requisito e fonte de moléculas angiogênicas capaz de conduzir a diferenciação de osteoblastos. Além disso, quando esses achados são considerados em conjunto, eles podem explicar o sucesso do titânio como liga metálica em aplicações biomédicas e explicar os trabalhos pioneiros do Prof. Brabemark em relação a osseointegração.

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