ANDERSON MOREIRA GOMES

COMPORTAMENTO DE CÉLULAS ENDOTELIAIS E MUSCULAR SUBMETIDAS AO *SHEAR STRESS*: UM PANORAMA CELULAR E BIOQUÍMICO

BOTUCATU

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COMPORTAMENTO DE CÉLULAS ENDOTELIAIS E MUSCULAR SUBMETIDAS AO *SHEAR STRESS*: UM PANORAMA CELULAR E BIOQUÍMICO

Dissertação apresentada ao Programa de Biotecnologia do Instituto de Biociência de Botucatu – UNESP – Universidade Estadual Paulista "Júlio Mesquita Filho", como requisito parcial para a obtenção do título de Mestre em Biotecnologia.

Orientador: Prof. Willian Fernando Zambuzzi

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ERRATA

Na dissertação de mestrado intitulada "*Comportamento de células endoteliais e muscular submetidas ao shear stress: um panorama celular e bioquímico*", trabalho de autoria de Anderson Moreira Gomes, sob orientação do Prof. Dr. Willian Fernando Zambuzzi, cabe a seguinte ressalva:

Na página V, referente aos agradecimentos, onde se lê "A FAPESP (Processo 2016/22270-8) pelo suporte financeiro indispensável para a realização deste trabalho.", leia-se "A Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)/CAPES (Processo nº 2016/22270-8) pelo suporte financeiro indispensável para a realização deste trabalho. "

Botucatu, 15 de Julho de 2019

Anderson Moreira Gomes

Faça o teu melhor, na condição que você tem, enquanto você não tem condições melhores, para fazer melhor ainda!

Mario Sergio Cortella

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1. Células endoteliais. 2. Músculo liso vascular. 3. Epigenética. 4. Cisalhamento.

Palavras-chave: Célula de musculatura lisa; Células endoteliais; Epigenética; *Shear stress*.

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RESUMO

As células endoteliais (ECs) e células musculares lisas (AoSMCs) são os principais componentes celulares do endotélio. As interações entre estes tipos celulares desempenham funções na homeostase e na estrutura vascular. Como uma interface entre o sangue e a parede do vaso, as ECs ocupam um local único diretamente exposto ao *shear stress* (SS), a força mecânica de atrito lateral produzido pelo fluxo de sangue na membrana apical da célula endotelial, que pode influenciar o comportamento de ambas ECs e AoSMCs. Geralmente, AoSMCs não sofrem diretamente às forcas de cisalhamento, no entanto, estas são diretamente expostas ao fluxo sanguíneo quando ocorre alguma injúria vascular, como por exemplo em algumas lesões ateroscleróticas ou por técnicas invasivas, como a angioplastia. As forças hemodinâmicas influenciam as propriedades funcionais do endotélio, porém estas não são profundamente compreendidas quanto aos mecanismos bioquímicos de respostas de células endoteliais e de musculatura lisa. Assim, a proposta desta dissertação foi estabelecer um modelo de cultivo in vitro que mimetize as forças tensionais de cisalhamento (shear stress), buscando compreender mecanismos celulares, bioquímicos e epigenéticos. Cultura de células primárias endoteliais e de musculatura lisa humanas foram obtidas da empresa LONZA e mantidas conforme recomendações do fabricante. Estas células foram mantidas rotineiramente em condições convencionais em incubadora de CO_2. Para mimetizar o fluxo sanguíneo, estas células, separadamente, foram semeadas em anel periférico de uma placa de petri (100mm de diâmetro), modificada de acordo estudos anteriores, e mantidas sob fluxo laminar por 72 horas, quando as células foram coletadas e encaminhadas para as diferentes metodologias. Nossos resultados estão apresentados em 2 capítulos nessa dissertação, sendo o Cap 2 dedicado aos mecanismos envolvidos na adaptação de células endoteliais ao shear stress, e o Cap. 3, dedicado aos mecanismos desencadeados em células musculares lisa. De um modo geral, nossos resultados mostram respostas diferenciais entre células endoteliais e de musculatura lisa ao shear-stress, as quais são regidas por mecanismos epigenéticos no controle da expressão gênica desencadeados pelo remodelamento da matriz extracelular, avaliada aqui como um mecanismo importante de adaptação destas células às forças de cisalhamento.

Palavras-Chave: Células endoteliais; Célula de musculatura lisa; Epigenética; *Shear stress.*

ABSTRACT

Endothelial cells (ECs) and smooth muscle cells (AoSMCs) are the major cellular components of the endothelium. Interactions between these cell types play roles in homeostasis and vascular structure. As an interface between the blood and the vessel wall, ECs occupy a single site directly exposed to shear stress (SS), the mechanical lateral friction force produced by blood flow on the apical membrane of the endothelial cell, which can influence behavior of both ECs and AoSMCs. Generally, AoSMCs do not directly undergo shear forces, however, they are directly exposed to blood flow when vascular injury occurs, for example in some atherosclerotic lesions or by invasive techniques such as angioplasty or surgical endarterectomy. Hemodynamic forces influence the functional properties of the endothelium, but these are not deeply understood as to the biochemical mechanisms of endothelial cell and smooth muscle responses. Thus, the purpose of this dissertation was to establish an in vitro culture model that mimics the shear forces, seeking to understand cellular, biochemical and epigenetic mechanisms. Culture of human endothelial and smooth muscle cells were obtained from the company LONZA and maintained according to the manufacturer's recommendations. These cells were routinely maintained under standard conditions in CO_2 incubator. To mimic blood flow, these cells were separately seeded in a peripheral ring of a petri dish (100mm diameter), modified according to previous studies, and maintained under laminar flow for 72 hours, when the cells were collected and sent to the different methodologies. Our results are presented in 2 chapters in this dissertation, with Cap. 2 dedicated to the mechanisms involved in the adaptation of endothelial cells to shear stress, and Cap. 3, dedicated to the mechanisms triggered in smooth muscle cells. Overall, our results show differential responses between endothelial and smooth muscle cells at shear stress, which are governed by epigenetic mechanisms in the control of gene expression triggered by the remodeling of the extracellular matrix, evaluated here as an important mechanism of adaptation of these cells to shear forces.

Key words: Endothelial cells; Smooth muscle cell; Epigenetics; Shear-stress.

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LISTA DE ABREVIAÇÕES

5-hmeC - 5-Hidroxi Metil Citosina
5-meC - 5-Metil Citosina
ADP - Adenosina Difosfato
AKT/PKB- Proteína Quinase B
AoSMC - Células do Músculo Liso Aórtico Humano
ATP – Adenosina Trifosfato
CaCl2 – Cloreto de Cálcio
CDK - Quinase Dependente de Ciclina
CO_2 – Dióxido de Carbono
COX-2 – Ciclo Oxigenase 2
DAPI - 4',6'-diamino-2-fenil-indol
DNA - Ácido Desoxirribonucleico
DNMT - DNA Metiltransferase
EBM-2 - Meio Basal de Células Endoteliais-2
EC - Células Endoteliais
ECM/MEC - Matriz Extracelular
eNOS - Óxido Nítrico Sintase Endotelial
ERK - Proteínas Quinases Reguladas por Estímulos Extracelulares
FAK – Cinase de Adesão Focal
FAK - **proteína** quinase de adesão focal
GAPDH - Gliceraldeído 3-fosfato desidrogenase
GDP - Guanosina Difosfato
GTP - Guanosina Trifosfato
HDAC - Histonas Deacetilases
HUVEC - Células Endoteliais da Veia Umbilical Humana
ICAM-1 - Molécula de Adesão Intercelular 1
iNOs - Óxido Nítrico Sintase Induzível
MAPK – Proteína Quinase Ativada por Mitógeno
MCP-1 - Proteína Quimioatraente de Monócitos-1
miRNA - Micro RNAs Não Codificantes
MMP - Metaloproteinase de Matriz
MMP-2 - Metaloproteinase de Matriz 2
MMP-9 - Metaloproteinase de Matriz 9
mRNA – RNA Mensagerio
NF-kB – Fator Nuclear kappa B
nNOS - Óxido Nítrico Sintase Neuronal
NO – Óxido Nítrico
PBS - Tampão Fosfato-Salino
PCR - Reação em Cadeia da Polimerase
PDGF-B - Fator de crescimento derivado de plaquetas B
PDGF-B - Fator de Crescimento Derivado de Plaquetas-B
PDK1- Quinase 1 Dependente de Fosfoinositídeo
PI – Fosfatidilinositol
PI3K – Fosfatidilinositol 3-Quinase
PIP - Fosfatidilinositol-4-fosfato
PIP2 - Fosfatidilinositol-4,5-bisfosfato
PKC – Proteína Quinase C
PVDF - Difluoreto de Polivinilideno
qPCR - PCR quantitativo
RB - Proteína do Retinoblastoma

RNA - Ácido Ribonucleico
RT-qPCR - PCR quantitativo em Tempo Real
SAM - S – Adenosilmetionina
SDS - Dodecil Sulfato de Sódio
SDS-PAGE – Gel de Poliacrilamida para Eletroforese-Dodecil Sulfato de Sódio
SIRT-1 – Sirtuína 1
SmBM - Meio Basal de Células Musculares Lisas
SMC – Células Musculares Lisa
SS – *Shear Stress*
T4-BGT - T_4 ß-glicosiltransferase
TBS - Solução salina Tris tamponada
TBST – Twen 20 Solução salina Tris tamponada
TDG - Timina-DNA Glicosilase
TET - Ten Eleven Translocation
TGF -β1 - Fator de Crescimento Transformador beta 1
TIMP-1 – Inibidor 1 de Metaloproteinase
TRIS-HCl – TRIS – Ácido clorídrico
UDP-Glc - Glicosil da Difosfoglucose de Uridina
VEGF – Fator de Crescimento Endotelial Vascular
VEGFR1 – Receptor 1 do Fator de Crescimento Endotelial Vascular
VEGFR2 – Receptor 2 do Fator de Crescimento Endotelial Vascular

CAPÍTULO 1

INTRODUÇÃO E OBJETIVO

INTRODUÇÃO

As células endoteliais (ECs) e células musculares lisas (AoSMCs) são os componentes básicos da parede dos vasos sanguíneos. As interações entre essas células desempenham papéis significativos na homeostase da estrutura e função do vaso sanguíneo. Como uma interface entre o sangue e a parede do vaso, as ECs ocupam uma localização única, diretamente exposta ao shear stress (SS) induzida pelo fluxo sanguíneo, o que pode influenciar os fenótipos de ambas ECs e AoSMCs. Por definição, podemos considerar o endotélio o maior órgão endócrino do corpo humano com uma superfície total estimada em 350 m^2, constituído por uma camada única de células em forma de fuso que separa o sangue da parede vascular e do interstício (Pries, Secomb, and Gaehtgens 2000; Bahia et al. 2006). Sua primeira descrição foi por Malpighi em 1661 (Pearce 2007), entretanto a terminologia endotélio foi introduzida pelo anatomista alemão His, em 1865 (Persson 2015), para contrapor a "epitélio", combinando as palavras gregas "endon" e "thele" as quais subentendem-se como revestimento de características papilares, sendo esta terminologia utilizada até hoje para descrever o revestimento interno do sistema circulatório (Évora 1999). Desta forma, o tecido endotelial classifica-se como um tecido de revestimento pavimentoso simples, constituídos por células geralmente achatadas e dispostas em camada única em sua orientação.

Suas funções estão em constante progresso de compreensão e considera-lo hoje apenas como uma camada inerte, ou ainda uma interface ativa, como no passado, já ficou extremamente ultrapassado (Helena et al. 2001). Devido às inúmeras funções realizadas pelo endotélio, este tecido em especial necessita de uma eficaz capacidade de comunicação intercelular (Gutstein 2003). Sua localização estratégica e vasta distribuição favorece o desempenho de suas funções relacionadas à homeostase vascular (Furchgott and Zawadzki 1980; Michiels 2003), interagindo de modo específico com os diferentes tecidos.

Por mais de duas décadas, cientistas levantaram a hipótese do possível envolvimento de forças mecânicas que atuam nas funções do endotélio (Yamamoto et al. 2003). Devido ao fato de estarem localizados entre o fluxo sanguíneo e a parede vascular, funcionando dessa forma como uma barreira da monocamada celular.(Y. S. J. Li, Haga, and Chien 2005), as células endoteliais são constantemente expostas a um ambiente de estresse mecânico. As duas forças hemodinâmicas predominantes são a tensão de cisalhamento (shear-stress, SS) e o estresse cíclico devido ao estiramento da parede vascular pela pressão transmural (Ballermann et al. 1998a). A diferença básica entre essas ações é que a primeira age na superfície apical da célula deformando-as na direção do fluxo sanguíneo, enquanto a

última tende a deformar as células em todas as direções. O shear stress (SS), é tangencialmente direcionado para a superfície luminal do vaso sanguíneo e é gerado quando um fluido viscoso, como o sangue, flui sobre ele (Mazzag, Tamaresis, and Barakat 2003). Embora todo o vaso sanguíneo esteja sujeito a forças hemodinâmicas devido à pressão arterial ou venosa, o estresse de cisalhamento resultante do fluxo é sustentado principalmente pelas células endoteliais (Y. S. J. Li, Haga, and Chien 2005). O SS *in vitro* desencadeia rápida remodelação do citoesqueleto e da matriz extracelular, além de ativar cascatas de sinalização que regulam a angiogênese e pode ser um fator de risco para diversas doenças cardiovasculares, podendo também regular a produção de substâncias vasoativas, como o fator relaxante derivado do endotélio e prostaciclinas, regulando a permeabilidade e endocitose de outras proteínas. Considerando o quadro celular geral em resposta a forças mecânicas, o SS compartilha semelhanças com um quadro inflamatório, pois além das proteínas já citadas anteriormente, a ação dessa força desencadeia a liberação aguda de óxido nítrico e prostaciclina, ativação de fatores de transcrição do fator nuclear (NF) e ativação transcricional de genes, incluindo ICAM-1, MCP-1, fator tecidual, fator de crescimento derivado de plaquetas-B (PDGF-B), fator de crescimento transformador (TGF -B1), COX-2 e óxido nítrico sintase (eNOS) (Y. J. Li, Haga, and Chien 2005). Outras experiências também mostraram que o fluxo sangüíneo pode superregular os fatores de crescimento endotelial vascular (VEGF) (Goettsch et al. 2008; William Li et al. 2003), sendo esta regulação elevada no endotélio da aorta, mas não no endotélio da veia cava. em seguida, de diferentes formas em vasos arteriais e venosos (Maharaj et al. 2006), mas este fato ainda precisa ser melhor elucidado. Outro comportamento interessante gerado pelo feedback das células endoteliais à SS é a adaptação por meio de remodelamento estrutural e achatamento celular para minimizar a tensão de cisalhamento, desta forma as células se tornam mais aderentes ao seu substrato.

A intensidade e direção do SS não são uniformes dentro dos vasos, dependendo do ciclo cardíaco e da geometria vascular, nos ramos arteriais e nas curvaturas ocorre a separação e recirculação do fluxo sanguíneo, onde células endoteliais são expostas a forças mecânica de ações baixas e/ou oscilantes (Franzoni et al. 2016). Existem várias maneiras de simular o SS *in vitro*, uma das quais é o cone e a placa, neste modelo a mudança nos padrões de tesão na cultura de células pode ser feita usando um sistema no qual um cone de Teflon é posicionado no centro de uma cultura de células, a ponta do cone é então colocada no centro e gira produzindo os padrões de fluxo SS através da monocamada de células (Reinhart-King, Fujiwara, and Berk 2008). Outro método bem estabelecido por dela

Paz em 2012, utiliza o modelo de estresse de cisalhamento baseado na rotação orbital do meio de cultura utilizado em uma monocamada de células endoteliais. Neste modelo, uma placa de cultura de 100 mm (diâmetro) é modificada inserindo no seu centro outra placa de cultura menor de 60 mm, criando assim um anel periférico e restringindo a adesão celular apenas nesta região da placa, garantindo assim que as células expostas à experiência estejam uniformemente sofrendo influência do SS (dela Paz et al. 2012).

Os processos de mecanotransdução celulares que ocorrem em resposta a estímulos físico-mecânicos, transformando-os em sinais bioquímicos, resultam em uma resposta fisiológica (Q. Zhang 2005). A conversão da mecanotransdução do shear-stress é decodificada pelas células endoteliais em etapas sequenciais iniciadas pela deformação da superfície celular pelo fluxo sanguíneo, desencadeando a transmissão intracelular do sinal para a conversão da força mecânica em atividade bioquímica e cascatas do segundo mensageiro que atuarão no fluxo sanguíneo (Califano and Reinhart-King 2010). Assim, o fluxo nos vasos sanguíneos gera o SS influenciando no desenvolvimento de fenótipos endoteliais associados principalmente à vasoproteção (Passerini et al. 2004). O SS ativa várias vias de transdução envolvendo respostas relacionadas à quinase de adesão focal (FAK), GTPases da família Rho, PI3-quinase, Ras-ERK, proteínas quinases ativadas por mitógeno (MAPKs), proteína quinase C (PKC) e fator nuclear-kB (NF-kB) porque parece ser sentida por um conjunto de macanotransdutores localizados na membrana celular, desencadeando consecutivas cascatas de sinalização nas células endoteliais (EC) que alteram as funções e expressões gênicas(B. D. Johnson, Mather, and Wallace 2011; Chatterjee and Fisher 2014).

As forças hemodinâmicas atuantes no endotélio exercem um papel fundamental para o desenvolvimento e progressão de algumas patologias vasculares, dentre as mais relacionadas estão a hiperplasia intimal (HI) e a aterosclerose (Heo, Fujiwara, and Abe 2014). Os estímulos mecânicos atuantes nas EC geram estímulos que são transformados em sinais biológicos que desencadeiam atividades proliferativas, migratórias e anti-apoptóticas das células musculares lisas presentes na camada média dos vasos sanguíneos, levando eventualmente ao IH (Y. Zhang et al. 2016). A aterosclerose é uma doença arterial inflamatória crônica na qual as placas se desenvolvem em regiões onde o fluxo é perturbado devido à geometria do vaso (Nigro, Abe, and Berk 2011), estabelecendo estrangulamento da região luminal. Regiões de bifurcações arteriais, curvas ou válvulas, locais onde o fluxo sanguíneo é menor e pode sofrer mudanças de direção (chamado de fluxo perturbado), tornar-se mais suscetível à formação de lesões ateroscleróticas, além de oclusões de

aterosclerose, gerando mais fluxo também afetam a progressão da lesão (Baeyens et al. 2016). O conhecimento da biomecânica das células endoteliais em resposta ao SS ajudará na compreensão do mecanismo, não apenas do crescimento e remodelamento dos vasos dependentes do fluxo sanguíneo, mas também da aterogênese e outras alterações vasculares.

Baseado nestes aspectos, torna-se evidente que o tecido endotelial não controla apenas a homeostase vascular, mas atua também em outros aspectos importantes, contribuindo, sobretudo, para mecanismos endócrinos entre tecidos, transportando células e moléculas, interferindo no crescimento, metabolismo, migração, diferenciação e morte de celular (Bahia et al. 2006).

Células endoteliais

As células endoteliais apresentam-se justapostas, com pouca substância extracelular com orientação seguindo a direção do fluxo sanguíneo. A superfície de contato entre as células e o tecido conjuntivo subjacente é denominada lâmina basal, sendo esta composta predominantemente por colágeno tipo IV, proteoglicanas e as glicoproteínas laminina e entactina (Bringel 2011). Entre as funções exercidas pela lâmina basal, podemos citar além de seu papel estrutural, seu envolvimento na regulação da proliferação e diferenciação celular, sua influência na polaridade e metabolismo celular e na transdução de sinais (Junqueira & Carneiro, 2008). Apesar das células endoteliais responderem bem a estímulos químicos agonistas que estimulam os receptores acoplados a proteína G, estas também respondem a forças mecânicas produzidas pelo fluxo sanguíneo, sintetizando e secretando fatores químicos envolvidos em numerosos processos fisiológicos, dentre os quais podemos citar a coagulação, fibrinólise, angiogênese e a manutenção do tônus vascular (Matlung, Bakker, and Vanbavel 2009).

Como estímulos químicos, podemos relacionar: os neurotransmissores acetilcolina e noradrenalina, o ATP e substância P; hormônios circulantes como catecolaminas, vasopressina, angiotensina II e insulina; os autacóides, bradicinina, histamina, ADP e endotelina e os produtos provenientes da coagulação sanguínea como a serotonina e a trombina (Matlung, Bakker, and Vanbavel 2009). Dentre as forças mecânicas, destacamos a tensão de complacência da parede vascular, a pressão hidrostática do conteúdo sanguíneo no interior do vaso e o *shear-stress,* promovida pela

força de atrito lateral exercida pelo fluxo sanguíneo sobre a membrana apical das células endoteliais (**Fig.1**) (Haga et al. 2003; Tineli et al. 2007).

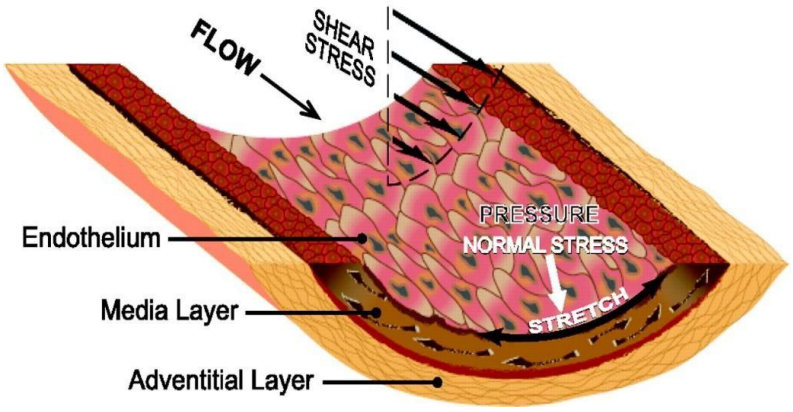

Figura 1: Diagrama esquemático mostrando a geração do shear stress (paralelo a superfície das células endoteliais) pelo fluxo sanguíneo e a geração da tensão normal stress (perpendicular à superfície das células endoteliais) e o estiramento circunferencial devido a ação da pressão (Chiu and Chien 2011).

Assim o fluxo sanguíneo destaca-se como o mediador mais importante destas funções exercidas pelas células endoteliais através dos processos de mecanotransdução endotelial. Desta forma, destacamos as células endoteliais como elemento chave na regulação da conversão de tensões mecânicas em respostas bioquímicas.

Células musculares lisas

As células musculares lisas modulam o tônus dos vasos sanguíneos **(Fig.2)** e, portanto, são os principais reguladores da pressão arterial. Por outro lado, o músculo liso tem que se adaptar às mudanças na pressão dos vasos e, consequentemente, as células musculares lisas (AoSMCs) exibem notável plasticidade e podem sofrer alterações fenotípicas em resposta a sugestões do ambiente local (Pfaltzgraff & Bader, 2015). É importante ressaltar que, embora a reação do endotélio à estimulação mecânica seja bem compreendida (Garcia-Cardena & Slegtenhorst, 2016), os processos moleculares que conduzem a adaptação das AoSMC às forças hemodinâmicas permanecem em grande parte obscuros e é justo dizer que a elucidação desses mecanismos permanece uma de grandes questões pendentes na biologia vascular. Estudos relatam que as células

musculares lisas respondem ao estresse hemodinâmico pela diminuição da sinalização de sobrevivência que pode culminar em apoptose e perda celular (Apenberg, Freyberg, & Friedl, 2003; Fitzgerald et al., 2008) e pode haver reciprocidade importante entre células musculares lisas e o endotélio, como por exemplo, sinais derivados do endotélio mediado por fluxo podem promover a produção de miócitos do fator de crescimento endotelial vascular - A (Uchida et al., 2015). Clinicamente, as AoSMCs podem estar diretamente expostas ao estresse de cisalhamento após a lesão endotelial que pode ocorrer durante a angioplastia. Estudos em lesões restenóticas que ocorrem após angioplastia coronariana, endarterectomia de carótida e enxerto de veia, sugerem que as células musculares desempenham um papel significativo na reestenose (Fitzgerald et al., 2008; Hao, Gabbiani e Bochaton-Piallat, 2003). Assim, o entendimento das respostas das AoSMCs ao shear stress é de grande interesse clínico e científico.

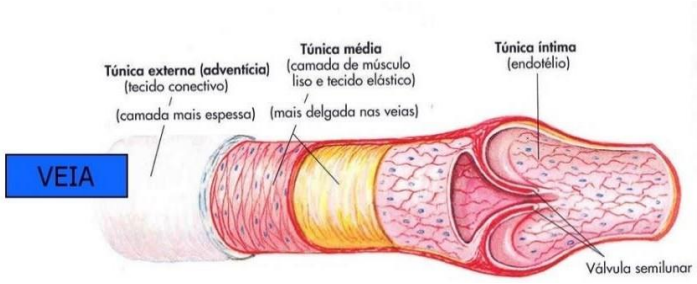

Figura 2: Estrutura interna da veia mostrando túnica externa composta basicamente por tecido conjuntivo, túnica média formada por musculares lisas e a túnica íntima constituída por células endoteliais.

Modelo *shear stress* (tensão de cisalhamento)

A conversão da mecanotransdução da tensão de cisalhamento é decodificada pelas células endoteliais em etapas sequenciais iniciada pela deformação da superfície celular pelo fluxo sanguíneo, desencadeando assim a transmissão intracelular do sinal para a conversão da força mecânica em atividade bioquímica e cascatas de segundos mensageiros que irá atuar na conversão da sinalização disparada em atividade efetora e no retro controle dos mecanismos de mecanotransdução (Califano and Reinhart-King 2010). Desta forma, a circulação arterial a tensão de cisalhamento esta implicada no desenvolvimento de fenótipos endoteliais associados à principalmente vasoprotecção (Passerini et al. 2004). Apesar de todo o vaso sanguíneo estar sujeito a forças hemodinâmicas devido a pressão arterial, a tensão de cisalhamento, resultante do fluxo

sanguíneo é suportado principalmente pelas células endoteliais, ao passo que as células de musculatura lisa são primariamente submetidas ao alongamento cíclico resultante da pressão pulsátil (**Fig.3**) (Y. S. J. Li, Haga, and Chien 2005).

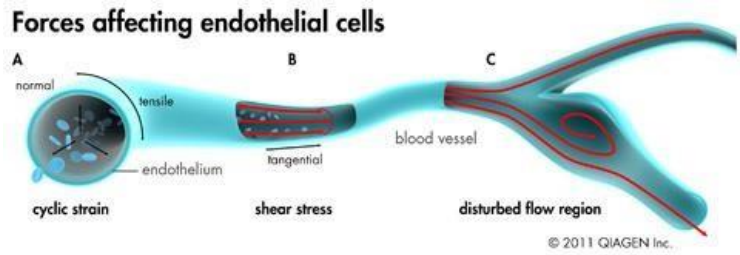

Figura 3: As células endoteliais são expostas a duas grandes forças: tensão cíclica (A) e shear stress do fluido (B). (C) ECs em áreas de alta curvatura ou jusante das bifurcações (seio carotídeo, bifurcação coronária) são expostos a shear stress perturbado (oscilatório com baixo tempo médio de shear stress).

Evidências mostram que o crescimento das células endoteliais pode ser afetado pela de tensão cisalhamento a que estas células são expostas. Pesquisas inicias realizadas por Levesque, Nerem, e Sprague em 1990, revelaram que uma SS laminar leva a uma diminuição da taxa de proliferação celular. Posteriormente, o estudo de Yamane et al. em 2010 mostrou que células endoteliais expostas a um fluxo laminar por um longo período de tempo apresentam taxas de síntese de DNA inferiores as células em condições estáticas. Estudos realizados por Lin et al. 2000 e Fitzgerald et al. 2008 revelaram que o fluxo laminar é capaz de reduzir o número de células que entram em no ciclo de reprodução celular, com a maioria das células ficando estagnadas nas fases G0 e G1 do ciclo. Em contraste a esses estudos, os experimentos de Passerini et al. (2004) e Levesque, Nerem, e Sprague (1990) mostraram que células endoteliais submetidas a um fluxo turbulento possuem taxas mais elevadas de proliferação quando comparadas a condições estáticas. Segundo Chiu e Chien em (2011) a taxa de síntese de DNA é significativamente mais elevada na vizinhança onde o fluxo laminar atua do que na própria área do fluxo. Os estudos de Y. S. J. Li, Haga, and Chien em 2005 mostraram que fluxos pulsáteis direcionados para frente inibem a proliferação de células endoteliais assim como ocorre em fluxos estacionários. Esses mesmos estudos também revelaram que um fluxo oscilatório, sem direcionamento, tem efeito semelhante ao fluxo turbulento no aumento da proliferação.

O mecanismo molecular pelo qual a tensão de cisalhamento laminar causa o impedimento da proliferação das ECs, tem se mostrado estar envolvido com o supressor tumoral p53, o qual induz o $P21^{cip}$ inibidor de Cdk (Y. S. J. Li, Haga, and Chien 2005). Segundo estudos de Shigeo Akimoto, Masako Mitsumata, Toshiyuki Sasaguri e 2000, a tensão de cisalhamento também provoca um aumento da associação de Cdk2 a $P21^{cip}$, o que resulta na diminuição da atividade da Cdk-quinase e da fosforilação da proteína do retinoblastoma (Rb) (Lin et al. 2000). A Rb é uma fosfoproteína nuclear que se liga ao DNA para regular a progressão do ciclo celular de muitos tipos de células por meio de seu estado de fosforilação (Bartek, Bartkova, and Lukas 1996). A fosforilação da Rb, que acorre quando sua serina e treonina são reduzidas, sofre varrições periódicas durante o ciclo celular, com predominância durante as fases Go e G1. Quando hiperfosforilada, a Rb consegue se ligar e inibir vários fatores de transcrição essenciais para a síntese de DNA, impedindo dessa forma a continuidade do ciclo celular (Nevis, Cordeiro-Stone, and Cook 2009).

Em células de musculatura lisa, por sua vez, as demais forças hemodinâmicas afetam a proliferação e a migração tanto em experimentos *in vivo* quanto *in vitro*, porém os efeitos diretos da tensão de cisalhamento ainda não estão totalmente compreendidos (Haga et al. 2003). Apesar das células endoteliais serem o tipo celular responsável pela decodificação da mecanotransdução promovida pela tensão de cisalhamento, está força hemodinâmica é importante na estimulação da proliferação e a migração das células musculares lisas após lesão endotelial (Asada et al. 2005). Ainda a tensão de cisalhamento pode afetar a hiperplasia neointimal e a proliferação das células musculares lisas em enxertos de próteses vasculares como a morfologia celular em enxertos venosos (Haga et al., 2003).

A partir de modelos de fluxo laminar, tem sido demostrado que a tensão de cisalhamento pode aumentar a expressão do fator de crescimento de fibroblasto e da enzima conversora de angiotensina. Da produção de fator de crescimento transformante β1, manofosfato de guanosina cíclico, ativador de plasminogênio tecidual e oxido nítrico e da liberação do fator de crescimento de fibroblasto 2, prostaglandinas E2 e I2, além de aumentar a migração de células da musculatura lisa (Haga et al., 2003). Por outro lado, em células endoteliais mostraram ser capazes de induzir a proliferação, auxiliar na reorganização do citoesqueleto, aumentar a produção de fator de crescimento, interferir na transdução de sinal da via PI3K, ativa as vias pp70 e proteína quinase ativadas por mitógenos, além de estimular a síntese de DNA (Bakker and Gans 2000; Dardik et al. 2005). Desta forma, podemos destacar a importância das forças hemodinâmicas geradas

principalmente pelo fluxo sanguíneo para a manutenção da homeostase da rede de vasos do sistema cardiovascular e em especial a tensão de cisalhamento a qual está intrinsecamente relacionada a remodelagem da parede do vaso sanguíneo, tanto em processos fisiológicos como também na patogênese de doenças cardiovasculares, como a aterosclerose e reestenose (Sweeney 2004).

Remodelamento da matriz do tecido endotelial

Estudos trazem evidências de que o processo de remodelagem da parede dos vasos não depende somente da ação de forças hemodinâmicas, mas também (talvez em função de) de outros fatores, como a ativação enzimática de metaloproteinases de matriz (MMPs). Estas enzimas habilitam a remodelagem vascular por meio da destruição da arquitetura da matriz extracelular existente enquanto uma nova matriz é sintetizada e organizada (Bronneberg 2003). Estas enzimas fazem parte de uma família de proteases dependentes de zinco, capazes de clivar numerosos substratos pericelulares, incluindo outras proteinases, fatores de coagulação, receptores de superfície celular, fatores de crescimento e praticamente todos os componentes da membrana basal e da matriz extracelular. Assim, em virtude da sua capacidade de modificar uma grande variedade de peptídeos bioativos, as MMPs influenciam diretamente em diversos processos fisiológicos e patológicos (Sternlicht and Werb 2001).

Pesquisas com relação a regulação das MMPs nos vasos sanguíneos mostraram a existência de uma conexão entre estímulos mecânicos (tensão de cisalhamento) e a transcrição, secreção e ativação de MMPs, em especial MMP-2 e MMP-9, as principais responsáveis pelo processo de degradação da matriz extracelular (Palumbo et al. 2000; Sho et al. 2002; Chatzizisis et al. 2007). Testes *in vivo* realizados em roedores mostraram que, após indução de inflamação e inicial formação de trombos foi observado aumento da expressão de MMP-2 e MMP-9 durante o processo de reconstrução da matriz extracelular (Deatrick et al. 2005; Bergan, Pascarella, and Schmid-Schönbein 2008). Cabe ressaltar que a expressão de MMP-2 mostrou-se aumentada principalmente nas camadas sub-íntima e média do vaso, enquanto a MMP-9 foi mais expressa nas camadas íntima e sub-íntima (Bergan, Pascarella, and Schmid-Schönbein 2008), camadas estas onde estão presentes células endoteliais e células musculares lisas, foco de nosso estudo. Ainda, estudos *in vitro* em que expuseram EC a uma baixa SS e os resultados mostraram um aumento na expressão das MMPs (Sakamoto et al. 2009). Em contraste, zimografia revelou que a

exposição à alta tensão de cisalhamento, reduziu a secreção de MMP-2 em células endoteliais (Yamane et al. 2010). Além disso, um estudo atual envolvendo células tronco derivadas da medula óssea submetidas a alta SS também apresentou expressões elevadas das metaloproteinases (Ueng et al., 2016).

Mecanismos epigenéticos

Recentemente com os avanços tecnológicos na área de biologia molecular e sequenciamento gênico, fortes evidencias têm sido fornecidas para o papel da epigenética em diferentes processos biológicos. O termo epigenética foi introduzido inicialmente por Conrad Waddington no ano de 1940 para descrever as interações dos genes com o ambiente e sua relação com o fenótipo (Wu Ct and Morris 2001; Dupont, Armant, and Brenner 2009; Waddington 2012).

Os mecanismos epigenéticos desempenham um papel fundamental nas funções celulares contribuindo nos processos de desenvolvimento além de influenciarem nas redes de comunicação e sinalização celulares. Assim, a manutenção do código epigenético é crítica tanto para a realização dos processos celulares básicos como também processos mais complexos como a diferenciação e reparo ósseo (Eslaminejad, Fani, and Shahhoseini 2013). Entre os principais mecanismos de regulação epigenética, destacam-se as modificações químicas no DNA, como metilação e hidroximetilação de citosinas, modificações pós-translacionais das histonas (metilação, ubiquitinação, acetilação e fosforilação) e os micro RNAs (miRNAs) não codificantes (Tost 2010), em mecanismos pos-transcricionais.

A metilação do DNA é um mecanismo epigenético, guiado pelas enzimas DNA metiltransferases (DNMTs), que consiste na adição covalente de um grupamento metil (CH_3), doado pelo substrato S-adenosilmetionina (SAM), ao carbono 5′ de uma citosina que geralmente precede uma guanina (dinucleotídeo CpG, conhecido tamém como ilha CpG), formando a 5-metil citosina (5-meC) (Sawan et al. 2008). Em mamíferos foram identificados cinco membros de DNMTs, as DNMT1, 2, 3A, 3B e 3L, divididas em dois grupos. O primeiro grupo é formado pela DMNT1, que é denominada DNMT de manutenção, por apresentar alta afinidade por fitas de DNA hemimetilado e, por este motivo, é responsável pela manutenção dos padrões de metilação do DNA durante a replicação. O segundo grupo, as DMNTs *de novo*, é representado pela DNMT3A e 3B, responsáveis pela ocorrência das ondas sequenciais de metilação de novo em regiões do genoma sem nenhuma indicação prévia de

metilação (Attwood, Yung, and Richardson 2014). Em contrapartida, o padrão de metilação do DNA pode ser perdido tanto passivamente, devido à inibição da atividade da DNMT de manutenção ou, por processo ativo, quando a 5-metilcitosina é removida enzimaticamente. Embora os mecanismos de metilação do DNA estejam relativamente bem caracterizados, o mecanismo de desmetilação ainda não foi totalmente elucidado (Gehring, Reik, and Henikoff 2009). Neste sentido, os estudos apontam para uma família *Ten-Eleven Translocation* (TET). A família TET em mamíferos é composta por 3 membros, TET1, 2 e 3, os quais compartilham um elevado grau de homologia no seu domínio catalítico (Loenarz and Schofield 2009). Estas proteínas são capazes de oxidar a citosina metilada (5-meC) e catalisar a conversão da 5-meC em 5-hidroximetilcitosina (5-hmeC) formando também a 5-formilcitosina e 5-carboxilcitosina para serem posteriormente reconhecidas e eliminadas por vias enzimáticas distintas (Dao et al. 2014). No entanto, o real mecanismo de desmetilação ainda não foi totalmente elucidado e estudos sugerem vias ou mecanismos alternativos que também são regulados pela dinâmica dos eventos de desmetilação. Contudo, os estudos concordam que a oxidação inicial de 5meC para 5-hmeC pelas TETs é um pré-requisito indispensável para os processos subsequentes de desmetilação do DNA, independentemente da forma como estes passos finais serão mediados (**Fig.4**) (Maiti and Drohat 2011).

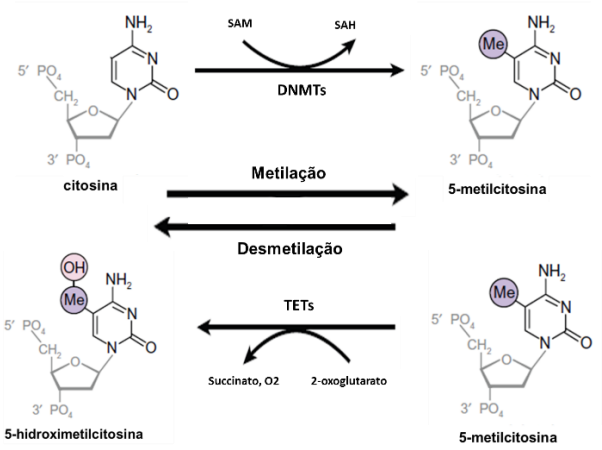

Figura 4: Mecanismo de metilação e desmetilação ativa do DNA. O esquema representa a transferência do grupo metil a citocina pela ação das DNA metiltransferases (DNMTs) e sua remoção pela ação das desmetilases, proteínas pertencentes à família das proteínas de translocação (TETs).

Em eucariotos, a metilação do DNA ocorre predominantemente em regiões repetitivas do genoma que possui abundância de ilhas CpG. Em geral, os dinucleotídeos

CpG são relativamente raros no genoma dos mamíferos, ocorrendo normalmente em regiões densas chamadas ilhas CpG localizadas principalmente em regiões promotoras de genes funcionais (I. T. Johnson and Belshaw 2008). A metilação dos CpGs em regiões promotoras ou no primeiro éxon de um gene pode modular e/ou significantemente silenciar a expressão gênica direta ou indiretamente (Turek-Plewa and Jagodziński 2005). No mecanismo direto, a metilação nos CpGs altera acessibilidade do DNA aos fatores de transcrição; por outro lado, o mecanismo indireto é mediado pela ação de proteínas (meCpGs) que possuem afinidade e reconhecem 5-metilcitosina em ilhas CpG, bloqueando assim a ligação dos fatores de transcrição ou por proteínas que formam complexos com outras proteínas de remodelamento de cromatina e estão envolvidas na estabilização da estrutura da heterocromatina (**Fig.5**) (Turek-Plewa e Jagodziski, 2005).

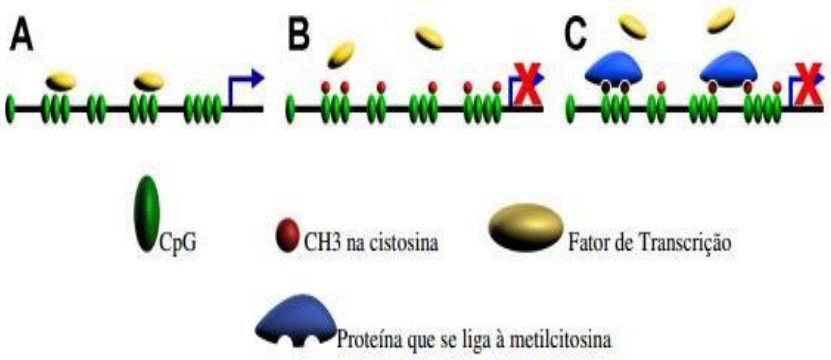

Figura 5: Mecanismo de inibição da transcrição gênica pela metilação de DNA. (A) Região promotora desmetilada permitindo a ligação dos fatores de transcrição; **(B)** Metilação impedindo a ligação dos fatores de transcrição e **(C)** Bloqueio da ligação dos fatores de ligação na região promotora pela ação das proteínas que se ligam à metilcitosina em ilhas CpG (meCpGs). **Fonte:** Attwood et al., 2002.

O estudo do tecido endotelial e das células musculares lisa adjacentes tem sido cada vez mais necessário, uma vez que atualmente tem sido apontado como tecido fundamental para uma série de eventos parácrinos, endócrinos e autócrinos, influenciando mecanismos biológicos sistêmicos. Nesta dissertação, como listado abaixo, nossa proposta foi buscar mecanismos celulares e bioquímicos de células endoteliais e musculares lisas submetidas ao modelo de Shear Stress, um modelo biológico capaz de mimetizar forças desenvolvidas pela ação do fluxo sanguíneo, associando com potenciais marcas epigenéticas envolvidas nestes mecanismos de adaptação celular.

OBJETIVO

Baseando-nos no contexto de biologia vascular, nosso objetivo foi compreender eventos bioquímicos e celulares envolvidos com o comportamento de adaptação de células endoteliais e de musculatura lisa ao *Shear Stress.* Nossos objetivos específicos estão agrupados em 2 artigos científicos, listados nos capítulos seguintes.

CAPÍTULO 2

ARTIGO

Possible compromising of ECM remodeling in driving epigenetic marks in shear-stressed endothelial cells.

Artigo submetido e em processo de *Major Revision* pelo *Journal of Cellular Physiology (*JCP-19-06-90)

Possible compromising of ECM remodeling in driving epigenetic marks in shear-stressed endothelial cells

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Keywords: Endothelial cell; Shear Stress; Mechanotransduction; Signaling; Epigenetic; Extracellular Matrix.

ABSTRACT

Modifications on shear-stress-based mechanical forces are associated with pathophysiological susceptibility and their effect on endothelial cells needs to be better addressed. This background prompted us to evaluate the biochemical and cellular effect of shear stress in human primary venous endothelial cells obtained from umbilical cord (HUVECs), using an *in vitro* model to mimic the laminar blood flow, reaching an intensity 1 to 4 Pa. Our data shows that shear-stress provokes profound cellular changes requiring ECM-remodeling as a pre-requisite to endothelial cell survival concomitant to a dynamic cytoskeleton rearrangement upon integrin activation. Additionally, these biochemical processes seem to be linked with a rigorous modification of transcriptional processing as indicated by the epigenetic signature, once global analyses of methylation (5-meC) and hydroxymethylation (5-hmeC) show a significant diminution of 5-hmeC in response to shear-stress, suggesting a global repression of genes transcription. Summarizing, our set of data reveals an important ECM remodeling in response to laminar shear-stress and this signal might triggers intracellular mechanism to drive epigenetic landscape. Altogether, these findings lead us to believe an influence of mechanotransduction on modifying the phenotype of endothelial cells, opening novel perspectives to understand the molecular basis of pathophysiological disorders related with vascular biology.

Keywords: Endothelial cell; Shear Stress; Mechanotransduction; Signaling; Epigenetic; Extracellular Matrix.

INTRODUCTION

It has been hypothesized the possible involvement of mechanical forces on endothelium functions (Ando and Kamiya 1993; Y. S. J. Li, Haga, and Chien 2005). To date, two predominant hemodynamic forces are shear stress (SS) and cyclic stress due to stretching of the vascular wall by transmural pressure (Ballermann et al. 1998b), it being that shear stress is tangentially directed on the luminal surface of the blood vessel (Mazzag, Tamaresis, and Barakat 2003). Although the entire blood vessel is subject to hemodynamic forces due to arterial or venous pressure, the shear stress resulting from the flow is supported mainly by the endothelial cells (Y. S. J. Li, Haga, and Chien 2005). Acute SS in vitro triggers rapid remodeling of the both cytoskeleton and extracellular matrix (Pinto et al. 2018), as well as activating signaling cascades that regulate angiogenesis and cardiovascular disorders, and may also regulate the production of vasoactive substances, such as prostacyclin and histamine, regulating the permeability and endocytosis of the endothelium (Tesfamariam and Cohen 1988; Busse, Hecker, and Fleming 1994). Considering the general endothelial cell picture in response to mechanical forces, the SS shares similarities with an inflammatory landscape mainly because SS releases nitric oxide (NO) and prostacyclin, activates of nuclear transcription factors and transcriptional of genes, including ICAM-1, MCP-1, tissue factor, platelet-derived growth factor-B (PDGF-B), transforming growth factor (TGF) -β1, COX-2 and nitric oxide synthase (eNOS) (Y. S. J. Li, Haga, and Chien 2005). Other experiments also showed that blood flow up-regulate vascular endothelial growth factors (VEGF) (Goettsch et al. 2008; M. Li et al. 2009), and this regulation is elevated in the endothelium of the aorta but not with significance on endothelium of the vein cava (Maharaj et al. 2006) – thus, it is clear that this physiological behavior needs to be better elucidated. Another interesting behavior resulting by the feedback of the endothelial cells to the SS is the adaptation to tensional forces requiring intense cytoskeleton rearrangement to better support cell adhesion onto substrate.

To better of our understanding, alternative methodologies to study cellular and molecular behavior of endothelial cells responding to SS are necessaries, as that proposed by dela Paz et al. (2012) – the authors mimic shear stress by using an orbital rotation into the cell culture incubator challenging monolayer of endothelial cells. We have earlier experienced this methodology with slight modifications to suggest epigenetic machinery in modulating TIMP1 expression in smooth muscle cells (da Silva et al. 2018) as well as to identify some signaling mechanism in endothelial cell responding to a circuit of tensional

forces (Pinto et al. 2018). Briefly, mechanotransduction comprehends cellular processes occurring in response to physical and mechanical stimuli, transforming them into biochemical signals culminating in a physiological response (Q. Zhang 2005). Shear-stress-based mechanotransduction is decoded by endothelial cells in sequential steps initiated by deformation of the cell surface by blood flow, thereby triggering the intracellular transmission of the signal for the conversion of mechanical force into biochemical activity and second messenger cascades that will act in the conversion of triggered signaling to effector activity and in the retro control of mechanotransduction mechanisms (Califano and Reinhart-King 2010). In addition, hemodynamic force acts on modulating endothelium dynamic during the development of some vascular disorders (such as intimal hyperplasia and atherosclerosis) (Heo, Fujiwara, and Abe 2014), reinforcing the importance on understanding the biology of endothelial cells (EC) in response to physiological forces promoted by the laminar shear stress, mainly looking for differential and drugable biomarkers to be further investigated in clinical trials related with blood flow disturbance.

Although some progress has been achieved on this sense, considering intracellular pathways involved with different endothelial phenotypes, such as adhesion and proliferative demands (B. D. Johnson, Mather, and Wallace 2011; Chatterjee and Fisher 2014), in this study we have focused on understanding the adaptive behavior of human venous-obtained primary endothelial cells subjected to SS, considering extracellular remodeling on modulating epigenetic signature. To date, recent reports demonstrate that tissue-specific gene expression is regulated via epigenetic mechanisms, including DNA methylation (Schilling and Rehli 2007). In vertebrates, methylation is catalyzed by DNA methyltransferase (Yan, Matouk, and Marsden 2010). Conversely, DNA demethylation induces transcription (Cimmino et al. 2011; J. U. Guo et al. 2011), and is regulated by ten-eleven translocation 1–3 (TET1-3), which oxidizes 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine. However, the mechanisms by which this balance occurs in laminar shear stressed endothelial cells have not been well established.

MATERIALS AND METHODS

1. Reagents and antibodies

Ripa buffer (R0278), Z-Leu-Leu-Leu-al (MG-32, C2211), Phosphatase inhibitor cocktail 2 (P5726), bovine serum albumin (A7906), gelatin (48723), saponin (47036), triton X100 (9284)

and agarose (A9539) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gotaq qPCR master mix (A6002) was purchased from PROMEGA (Madison, Wisconsin, EUA). DNase I (18068015), High-Capacity cDNA Reverse Transcription. Antibodies against GAPDH (#2118), Cofilin (#3212), phospho-Cofilin (Ser3) (#3311), α4-Integrin (#4600), β3-Integrin (D7X3P) (#13166), Rac1/Cdc42 (#4651), Akt (pan) (C67E7) (#4691), Phospho-Akt (Ser473) (D9E) (#4060), Src (36D10) (#2109), p38 MAPK (D13E1) (#8690), phospho-p38 MAPK (THR180/TYR182) (D3F9) (#4511), and P15 INK4B (#4822) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PI3K p85 [6G10] (ab189403) and anti-ERK1 + ERK2 (ab17942), were purchased from Abcam (Cambridge, MA, USA). Enzymes T4-β-glucosyltransferase (T4-BGT) (M0357S), MspI (R0106S) and HpaII (R0171S) was purchased from New England BioLabs, Beverly, MA. SYBR™ Safe DNA Gel Stain (S33102), UltraPure™ DEPC-Treated Water (750023) and Proteinase K (25530-015) was purchased from Thermo Fisher Scientific(Seattle, WA).

2. Cell culture

Human primary umbilical vein endothelial cells (HUVEC - CC-2571) were purchased from Lonza (Walkersville, MD) and used for experiments between passages 3 to 8. The cells were cultured in proper Endothelial Cell Basal Medium-2 (EBM-2) (LONZA, Walkersville, MD) supplemented with EGM™-2 SingleQuot Kit Suppl. & Growth Factors [0.5 mg/mL hEGF, 5 mg/mL insulin, 1 mg/mL hFGF, 50 mg/mL gentamicin/amphotericin-B, and 5% fetal bovine serum (Lonza, CC-4149)] at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were routinely maintained with fresh medium changed every 3 days.

3. Shear stress

The shear stress was performed in human primary umbilical vein endothelial cells (HUVEC) (10 x 10^4 cells) seeded in the periphery ring of modified 100-mm made by bonding the bottom of 60-mm culture dishes into the center of the 100-mm culture dishes using medical silicone and thereafter the dishes were sterilized using UV light for 15 min, as described by dela Paz with modifications (dela Paz et al., 2012). The cells were maintained in Endothelial Cell Basal Medium-2 (EBM-2) (LONZA, Walkersville, MD) and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced with DMEM containing 1% of SFB and antibiotics (Nutricel, Campinas, SP) and then confluent monolayers were subjected to orbital shear stress up to 72 hours at 37˚C in CO_2 incubator using an SK-O180-Pro Digital Orbital Shaker (SCILOGEX, Rocky Hill, CT, EUA). The cells

were subjected to shear stress protocols with a rotation frequency of 100 rpm that was chosen by prior calculating maximum stress subjected to the cells in according to the formula: $\tau max=\alpha\sqrt{\rho\eta}(2\pi f)$ 3 , where ρ= density and η= viscosity and α = radius. Considering our experimental condition, Pa.s and α =012M, resulting in a correspondent physiological shear stress (6-40 dynes/cm) (dela Paz et al., 2012). HUVEC exactly obtained the same passage, which were not subjected to shear stress, were kept in the same CO_2 incubator and were considered as static control.

4. Zymography analysis

The proteolytic activity of MMP-2 and MMP-9 present in challenged HUVEC-conditioned medium was assayed by gelatin zymography as described by Lefebvre (Lefebvre et al., 1991). Static and shear stressed cultures were properly challenged and the conditioned medium harvested, which was clarified by centrifugation at 14.000 rpm for 15 min at 4°C, and further stored at −20 °C. previously, the samples were quantified using the Lowry protein assay (LOWRY et al., 1951) and diluted in non-reducing buffer (0.1 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS and 0.001% (w/v) bromophenol blue). Equal amounts of protein (150 µg) were loaded onto SDS–polyacrylamide gel (10% (w/v)) and 4% (w/v) gelatin. MMPs renaturation was performed in 2% (v/v) Triton X-100 for over-night followed by incubation in incubation buffer [50 mM Tris–HCl and 10 mM CaCl2 (pH 7.4)] at 37 °C overnight. Afterwards, gels were stained with 0.5% (w/v) coomassie blue G 250 for 3 hours, washed in a 30% (v/v) methanol and 10% (v/v) glacial acetic acid solution until the bands appear and then analyzed using software ImageJ.

5. Western blot

HUVECs were subjected to shear stress protocol up to 72 hours, when they were washed in ice-cold PBS and protein extracts were obtained using a RIPA lysis buffer (Sigma Aldrich) supplemented with protease inhibitors (Sigma Aldrich) for 1 h on ice. Thereafter, protein extracts were cleared by centrifugation 14,000 rpm for 15 min at 4°C. The pellet was then resuspended in 100 µl of RIPA lysis buffer (Sigma) and the clarified protein extracts were used to measure protein concentration by Lowry method (Hartree 1972). An equal volume of 2-sodium dodecyl sulfate (SDS) gel loading buffer [100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue, and 20% glycerol] was added to samples and boiled for 5 min at 95°C. Aliquots of the samples (75-100 µg/lane) were resolved into SDS-PAGE (8, 10 or 12% gels) and after transferred to PVDF membranes

(Millipore, USA), which were blocked with 5% nonfat dry milk dissolved in Tris-Buffered Saline (TBS)-Tween-20 (0.05%) and then incubated overnight with appropriate primary antibody at 1:1.000 dilutions at 4°C. After 1x-washing in TBS–Tween-20 (0.05%) and 2X-washing in TBS, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs antibodies, at 1:2,000 dilutions (in all immunoblotting assays), diluted in blocking buffer for 1H. Immunoreactive bands were detected using Thereafter, Enhance Chemiluminescence (ECL, Pierce, USA).

6. Total mRNA isolation and RT-qPCR analysis

Challenged HUVECs were harvested and total RNA properly isolated using Ambion TRIzol Reagent (Life Sciences – Fisher Scientific Inc., Walthan, MA, USA) and treated with DNase I (Invitrogen, Carls-band, CA, USA). cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time PCR was carried out in a total of 10 μL, containing PowerUpTM SYBRTM Green Master Mix 2X (5μL) (Applied Biosystems, Foster City, CA, USA), 0.4 μM of each primer, 100 ng of cDNA and nuclease free H_2O. Results were expressed as relative amounts of the transcripts using β-Actin as reference gene (housekeeping gene), using the comparative CT method (ΔΔCt) (Livak and Schmittgen 2001). Primers and details are described in **Table 1**.

7. DNA extraction

Collecting cells were homogenized in 500 μL of DNA extraction buffer (10 mM Tris pH 3.0, 0.5% SDS and 5 mM EDTA), then 10 μL Proteinase K (20 mg / ml) was added and incubated overnight at 65 ° C without stirring. The next day, 1.0 mL of phenol-chloroform (pH 8.0) was added at room temperature. The phenol is organic and separates the sample into a liquid phase of the protein, which turns white and the phenol turns yellow. The tubes are shaken manually for 5 seconds to form a milky emulsion at room temperature. The samples were centrifuged for 15 minutes at 14.000 rpm. Collect the supernatant, only the transparent part, without touching the white part (precipitated protein) or the yellow part (phenol) and transfer to a new 1,5 ml tube properly identified. Add 500 μL chloroform and stir for 5 seconds, gentle inversion at room temperature and centrifuge for 15 min at 14.000 rpm. Collect the supernatant and transfer again to new 1.5 mL tubes. Add 1 ml of cold absolute alcohol and 150 μl of 3M sodium acetate pH 5.2 and shake gently. Incubate at -20° C overnight. Centrifuge for 15 minutes at 14000 rpm and discard the supernatant. Add 500 mL

of 70% ice cold ethanol to sterile water vortex lightly and centrifuge 15 minutes at 14.000 rpm. 14. Discard the supernatant and allow the pellet to dry naturally (40 minutes at room temperature) or about 10 minutes in the oven at 37°C. Resuspend the pellet in 50 µL UltraPure ™ DEPC-Treated Water, rest 16 hours at room temperature to complete solubilization after stocking the DNA at -20 ° C.

Table 1: Primer sequences and RTqPCR conditions.

Gene (ID)	Primer	5'-3' Sequence	Product size (pb)	Reaction's Conditions
DNMT1 (1786)	Forward	GAGCCACAGATGCTGACAAA	93	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GACACAGGTGACCGTGCTTA		
DNMT3A (1788)	Forward	AAGGAGGAGCGGCCAGAG	98	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GGATGGGGACTTGGAGATCA		
DNMT3B (1789)	Forward	GGGAGGTGTCCAGTCTGCTA	89	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GGCTTTCTGAACGAGTCCTG		
AKT (207)	Forward	GGACTCCCGTTTGCGCCAGT	676	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GACGCTCACGCGCTCCTCTC		
TET1 (80312)	Forward	TCATGGGTGTCCAATTGCTA	81	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GATGAGCACCACCATCACAG		
TET2 (54790)	Forward	GGACATGATCCAGGAAGAGC	59	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	CCCTCAACATGGTTGGTTCT		
TET3 (200424)	Forward	CCCACAAGGACCAGCATAAC	89	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	CCATCTTGTACAGGGGGAGA		
MMP2 (4313)	Forward	AGCTCCCGGAAAAGATTGATG	59	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	CAGGGTGCTGGCTGAGTAGAT		
MMP9 (4318)	Forward	CACGCACGACGTCTTCCA	17	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	AAGCGGTCCTGGCAGAAAT		
TIMP1 (7076)	Forward	CCGCAGCGAGGAGTTTCTC	60	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GAGCTAAGCTCAGGCTGTTCCA		
RECK (8434)	Forward	TGCAAGCAGGCATCTTCAAA	60	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	ACCGAGCCCATTTCATTTCTG		
PI3K (5290)	Forward	ACTCTCAGCAGGCAAAGACC	772	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	ATTCAGTTCAATTGCAGAAGGAG		
VEGF (7422)	Forward	TGCAGATTATGCGGATCAAACC	34	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGCATTCACATTTGTTGTGCTGTAG		
VEGFR1 (2321)	Forward	CAGGCCCAGTTTCTGCCATT	41	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TTCCAGCTCAGCGTGGTCGTA		
VEGFR2 (3791)	Forward	CCAGCAAAAGCAGGGAGTCTGT	40	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGTCTGTGTCATCGGAGTGATATCC		
eNOS (NOS3) (4846)	Forward	TATTTGATGCTCGGGACTGC	99	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	AAGATTGCCTCGGTTTGTTG		
nNOS (NOS1) (4842)	Forward	CTCCAGCCCCGGTACTACTC	31	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TTAGCCACGTGGAGCAGACT		
P15 (1030)	Forward	TACAGGAGTCTCCGTTGGC	72	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GTGAGAGTGGCAGGGTCTG		
β-ACTIN (60)	Forward	ACAGAGCCTCGCCTTTGC	39	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GCGGCGATATCATCATCC		

8. Epigenetic: analysis of 5-methylcytosine (5-meC) and 5-hydrymethylcytosine (5-hmeC)

For the 5-meC and 5-hmeC analysis, genomic DNA was initially treated with T_4-β-glucosyltransferase (T_4-BGT) (New England Biolabs, Beverly, MA, USA), adding glucose moiety to 5-hmC (gDNA) to distinguish amongst DNA methylation and hydroxymethylation. For each sample, three tubes containing 400 ng gDNA each were treated with 1X NE buffer, 40 mM UDP glucose, T_4-BGT (1 unit) to a final volume of 40 µL and incubated at 37°C for 1 h, followed by 10 min at 65°C. Then, samples were digested with *Msp*I or *Hpa*II restriction enzymes (New England BioLabs, Beverly, MA, USA) or H_2O (control) according to the manufacturer's instructions. After digestion, for global DNA methylation analysis 10 µL digestion reactions was electrophoresed on 0.8% agarose gel, stained with SYBR Gold and photographed and then analyzed using software ImageJ. The gene specific analyze, was carried 40 amplification out in a total of 10 µl, containing PowerUp™ SYBR™ Green Master Mix 2X (5 µl) (Applied Biosystems, Foster City, CA), 0.5 µM of each primer, of treated gDNA and nuclease free H_2O. Primers were designed on regulatory regions such as DNaseI hypersensitivity clusters sites, layered by histone modifications marks, CpG regions and transcription factors binding sites, with free primer design and analysis software and further analyzed for secondary structures and annealing temperatures by the Beacon Designer, Free Edition (http://www.premierbiosoft.com/). Sequences and chromosome location were confirmed by the *in-silico* PCR (https://genome.ucsc.edu/).

9. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical analyses were performed using analysis of variance unpaired t test or non-parametric analysis. A p value <0.05 was considered to be statistically significant. The software used was GraphPad Prism 7 (GraphPad Software, USA).

RESULTS AND DISCUSSION

In order to better mimic the environment of endothelium tissue suffering the effect of blood flow, primary endothelial cells were obtained and further subjected to physiological shear stress by adapting earlier published protocol (dela Paz et al. 2012; Pinto et al. 2018). To evaluate this mechanotransduction, we prompted to compare endothelial cells subjected or not to the shear stress (**Fig. 6**).

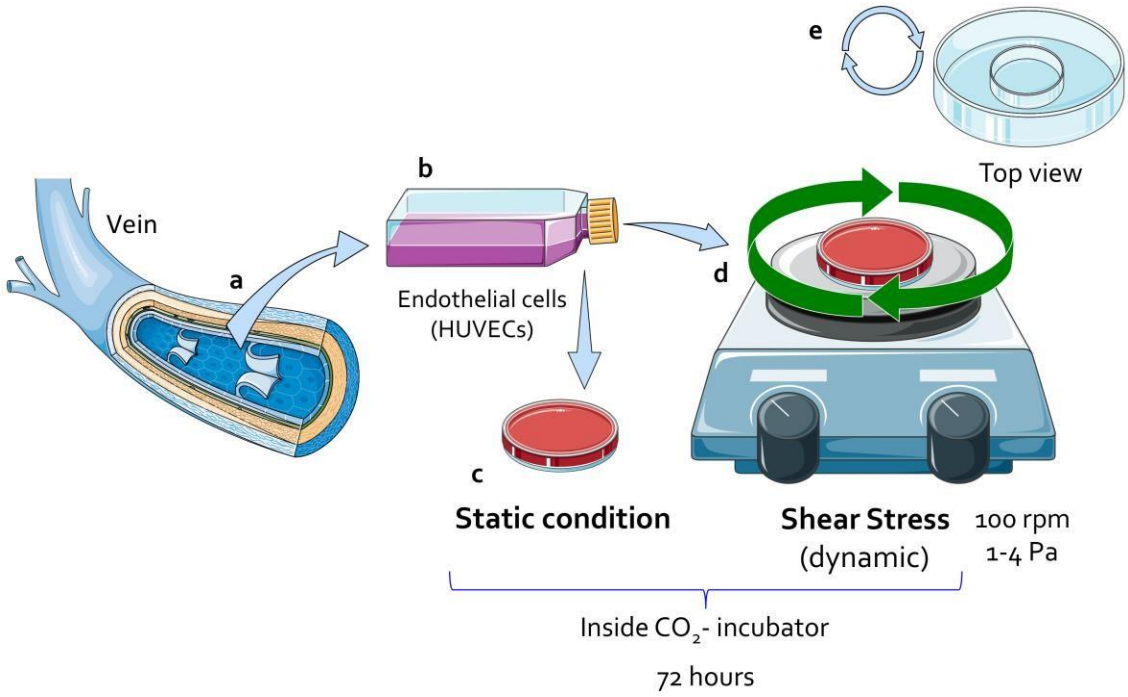

Figure 6: Outline of the experimental flow proposed in this study. (a) Schematic representation of the vascular endothelium depicting endothelial cells in the luminal compartment. The primary endothelial cells were obtained **(b)** and subjected to the shear stress **(d)** or maintained in classical condition (static) **(c)**. The top view of the modified petri dish to physiological mimicking blood flow is depicted in "**e**". To note, the shear stress was performed by using an incubator-adapted shaker to reach 100 RPM (1 - 4 Pa) up to 72 hours. The experiments were carried out in independent triplicates.

Shear stress provokes changes on molecular machinery governing survival and proliferation phenotypes intensifying cytoskeleton rearrangement.

Firstly, as PI3K/Akt is an important pathway to drive cell survival (Xie et al. 2018; Huang et al. 2018), we decided evaluating whether the behavior of this signaling was changed in response to shear stress. By exploring immunoblotting technology, we showed that PI3K was significantly up-regulated in mechanosignaling stimulus (**Fig. 7a**), while Akt was phosphorylated (**Fig. 7b**), and this pathway seems requiring the activation downstream of MAPK-Erk (**Fig. 7c**) and MAPK-P38 (**Fig 7d**). In fact, this axis of intracellular signaling seems being an important biomarker of laminar flow mainly because our data corroborates with others who have proposed this signaling in repressing endoplasmic reticulum stress-provoked apoptosis (J. C. Kim and Woo 2015; Liu, Chen, and Chen 2016; S. Kim and Woo 2018), and this molecular behavior might provides an atheroprotective effects of laminar flow. Earlier on this sense, studies have been reported the effected of laminar flow on Akt and MAPK (D. Guo, Chien, and Shyy 2007; Srivastava et al. 2017). Conversely, by using a very similar experimental model, we have proposed a circuit of tensional forces of the laminar flow and these variations does not affect MAPKs gene expressions (Pinto et al. 2018). Importantly to summarize, the **Fig. 7e** depicts on this intracellular signaling and suggests an effect requiring PI3K/Akt upon VEGFR activation.

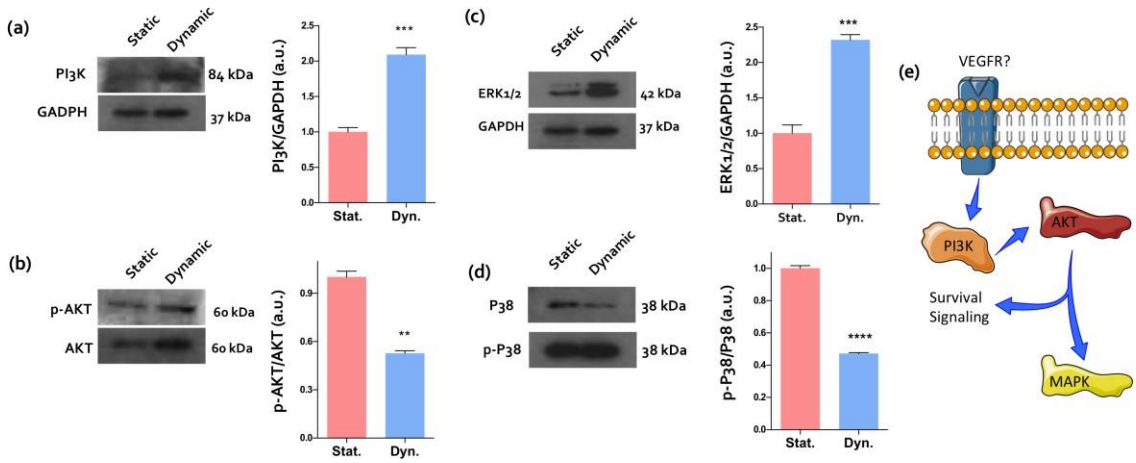

Figure 7: Mechanotransduction requires survival and proliferative pathways. The endothelial cells were subjected to the shear stress model up to 72H, when the cells were scraped out and properly lysed using RIPA buffer to further be resolved into SDS-PAGE and thereafter transferred in PVDF membranes. The membranes were incubated with different specific primary antibody respecting guidelines of western blotting technology. The proteins investigated were as follows: (**a**) PI3K (p=0.0002), (**b**) AKT and pAKT (p= 0.0021) and MAPKs (**c**) ERK (p= 0.0003) and (**d**) P38 and pP38 (p <0.0001). (**e**) Schematic depiction of the intracellular pathway evaluated in this stage. Representative blottings are shown, and the graphs represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control).

Thereafter, we reinforce the effect of shear stress on endothelial cell behavior by requiring an intense cytoskeleton rearrangement. Here, this effect was evaluated by the downstream signaling upon integrin activation (**Fig. 8f**) - we have shown that shear stress affects integrin expression, mainly considering the subunits β3 and α4 (**Fig 8a** and **b**, respectively). On this sense, Rac1 was significantly up-regulated (**Fig 8d**), while c-Src shows a discreet involvement (**Fig 8c**). Considering the end of this pathway, cofilin was significantly up-phosphorylated (>5-fold changes), reinforcing the cytoskeleton rearrangement as a crucial adapting cellular mechanism under shear stress disturbances. In conjunction, these findings in driving adaptive behavior to shear stress recapitulate the survival signaling required during cell adhesion (Willian Fernando Zambuzzi et al. 2009; Willian F. Zambuzzi, Milani, and Teti 2010; Bertazzo, Zambuzzi, Campos, Ogeda, et al. 2010; Bertazzo, Zambuzzi, Campos, Ferreira, et al. 2010; Willian F. Zambuzzi, Coelho, et al. 2011; Willian F. Zambuzzi, Ferreira, et al. 2011; Fernandes et al. 2014; Willian F. Zambuzzi et al. 2014; Baroncelli et al. 2018). To note, the signaling culminating on modulation of cofilin phosphorylation is majority pathway during β-actin rearrangement (Willian Fernando Zambuzzi et al. 2009; Silva et al. 2015; da Silva et al. 2018), and as actin peripherally contributes with the integrin roles, its seems sensate to require their involvement during adaptive effect in response to tensional forces.

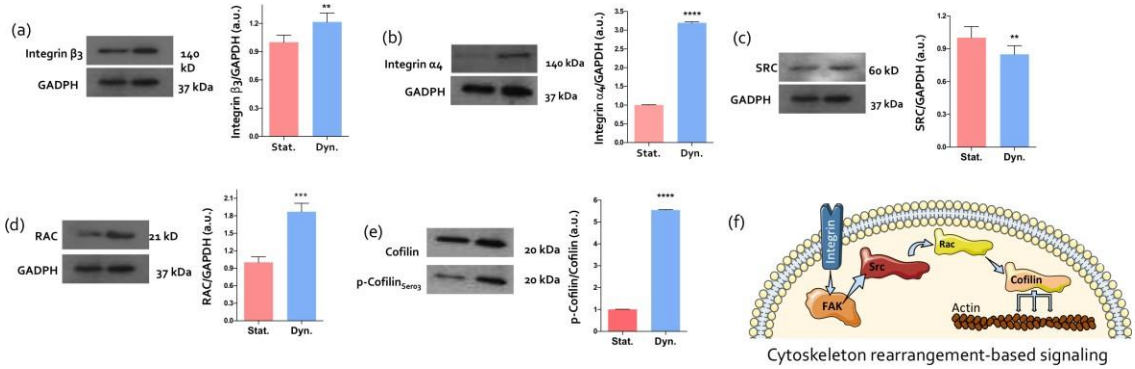

Figure 8: Shear stress promotes cytoskeleton rearrangement. The signaling cascade considered estimating cytoskeletal rearrangement is proposed in "**f**", where the pathway upon integrin activation culminates on cofilin phosphorylation, which is decisive to drive F-ctin polymerization, as show in the scheme. By using western blotting technology, the proteins investigated were as follows: (**a**) β3-Integrin (p=0.0379), (**b**) α4-Integrin (p<0.0001), (**c**) SRC (p=0.0139), (**d**) RAC (p=0.001), (**e**) Cofilin and p-Cofilin$_{Ser03}$ (p<0.0001). Representative blottings are shown, and the graphs represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control).

Additionally, all of those intracellular pathways were required to modulate endothelial cell growth in response to shear stress. Here, we show p15 was significantly up-expressed (**Fig 9b**; > 3 fold changes), while its respective transcripts profile remained unchanged (**Fig 9a**). This significant molecular processing of P15 prompted us to suggest a potential epigenetic influence on this experimental model *in vitro*, as it is shown later here.

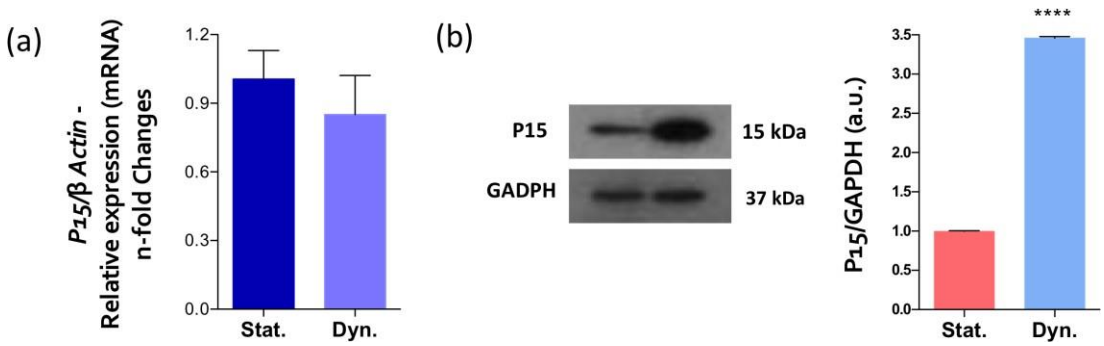

Figure 9: Potential effect of shear stress on proliferative phenotype was estimated by p15 evaluation. The challenged endothelial cells were obtained and the samples properly obtained. Although there are no significant changes on P15 transcript when compared with static-maintained endothelial cells (**a**) this is not reflected on the protein amount (**b**), when the challenged endothelial cells required an up-expression, it being over 3-fold changes higher that the control group (****p* <0,0001).

Finally, as VEGF signaling is knowingly involved in endothelial cell phenotype (L. Li et al. 2017; Heitzig et al. 2017) we decided evaluating VEGF (**Fig 10a**), VEGFR1 (**Fig 10b**) and VEGFR2 (**Fig 10c**) genes. Our data shows that although there is no effect on VEGF gene activation (**Fig 10a**), their both receptors were modified – while VEGFR1 was significantly repressed (**Fig 10b**), VEGFR2 was up-expressed (**Fig 10c**). In part, the **Fig 10c** reinforces the proposed illustration depicted in **Fig 7e**. In fact, VEGF, also known as vascular permeability factor, is known by being a key mediator of angiogenesis, promoting important endothelial events such as proliferation and migration (Zeng, Dvorak, and Mukhopadhyay 2001); to best of our knowledge, VEGF can be stated an important vasculoprotective molecule. In this sense, there are experimental evidences suggesting the angiogenic effect of VEGF being sustained by the formation of nitric oxide (NO), mainly considering that inhibitors of NO Synthase (NOS) have been reported to abolish the VEGF signaling (Morbidelli et al. 1996). Regarding this background, Bouloumié, Schini-Kerth, and Busse 1999 showed that VEGF induces an increase of eNOS mRNA. These findings led us to investigate the behavior of both isoforms of eNOS and nNOS genes in our proposed experimental model. Corroborating with those findings discussed previously, our data shows a significant involvement of eNOS (**Fig 10d**) and nNOS (**Fig 10e**), it being eNOS peaking over 15-fold changes increased when the endothelial cells were subjected to shear stress. The eNOS mRNA significances might be in response to the involvement of PI3K/Akt signaling, as suggested previously by others (Roviezzo et al. 2007).

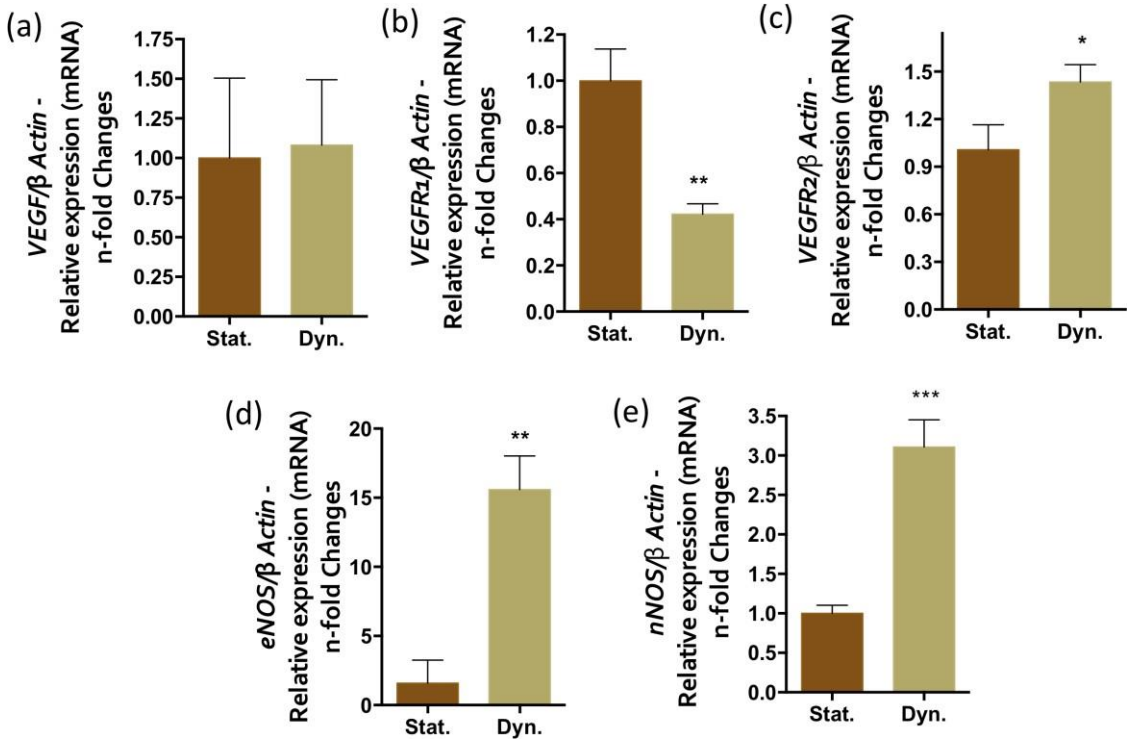

Figure 10: VEGFR2 and NOS are biomarkers of mechanotransduction. Behavior of endothelial cells phenotype is mediated by a wide range of families of growth factors and protease enzymes, such as VEGF and their membrane receptors, as well as isoforms of NOS enzymes. In order to evaluate their involvement in shear-stressed endothelial cells, the samples were obtained and the transcripts evaluated by RT-qPCR technology, as follows: (**a**) VEGF, (**b**) VEGFR1 (p= 0.0023), (**c**) VEGFR2 (p=0.018), (**d**) eNOS (p=0.0012) e (**e**) nNOS. The graphs bring the n-fold changes of the profile of transcripts normalized to the β actin, considered here a housekeeping gene.

Adaptive behavior of endothelial cells to shear stress requires ECM remodeling

As the dynamic extracellular matrix remodeling is a decisive mechanism to modulate cell survival signaling (Paiva et al. 2009; De Oliveira Demarchi et al. 2010; da Costa Fernandes, Ferreira, et al. 2018; da Costa Fernandes, Bezerra, et al. 2018), we investigated this mechanism in challenged endothelial cells by considering matrix metalloproteinases (MMPs) behavior as well as their endogenous inhibitors (**Fig 11a-d**). Our data shows a significant effect of SS on up-modulating MMP9 mRNA (**Fig 11b**). Thereafter, we further investigated the MMP2 and MMP9 activities by using zymography approach (**Fig 11e-i**) and our data reinforces the requirement of MMP9 on this scenario (**Fig 11e, f**). This biology of the ECM remodeling requiring MMP9 seems be a effect of VEGF, as revised by Ferrara and Davis-Smyth (1997) a mechanism meaning to supply oxygen to eventual ischemic tissue (Ferrara and Davis-Smyth 1997). In addition, ECM is the non-cellular compartment and provides mechanical scaffold for the cell adhesion and consequent survival signaling. The correct governance of ECM remodeling is necessary to drive angiogenic processes and mainly because this has been widely evaluated in cancer progression (Hamano et al. 2003). Briefly, antiangiogenic peptides have been listed to modulate MMPs activities and indeed promoting a negative endothelial cell proliferation control and concomitant inducing apoptosis-related signaling (Maeshima et al. 2001; Neve et al. 2014). Altogether, this knowledge provides potential basis for the development new therapeutic approaches against abnormal angiogenesis and lessons from shear stress-induced ECM remodeling in endothelial cells should be useful on this sense.

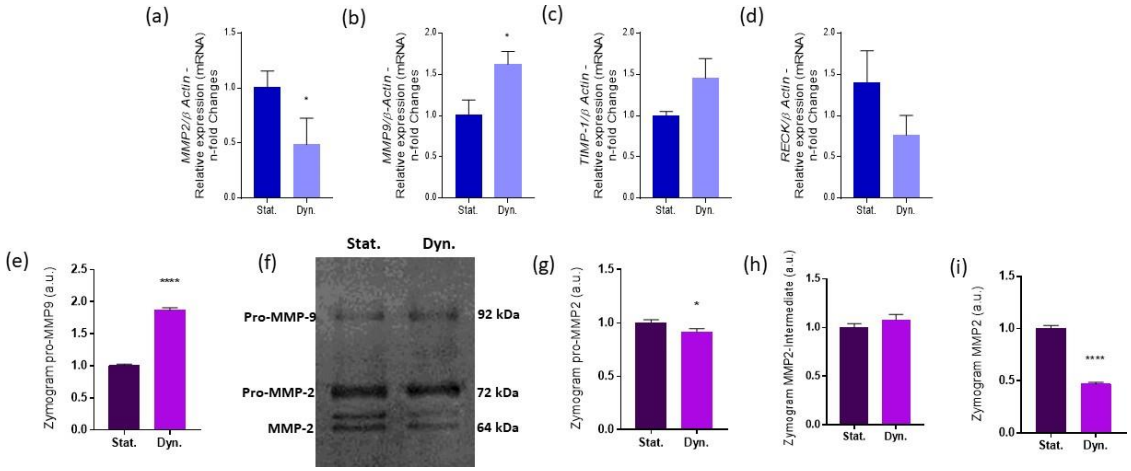

Figure 11: Extracellular matrix (ECM) remodeling was evaluated considering MMPs behavior. Firstly, the repertory of genes related with MMPs biology was considered here, as follows: MMP2 (**a**), MMP9 (**b**), TIMP1 (**c**), and RECK (**d**) – although with no differences on their endogenous inhibitors, MMP2 (p=0.0111) was overexpressed while MMP9 was repressed (p=0.0330). Thereafter evaluating gene expression, the activity of MMPs 2 and 9 were evaluated further by zymography approach by considering electrophoretic gel containing gelatin (4%). Conversely, our results show higher activity of proMMP9 than unchallenged cells (**e,f**; ****p <0.0001), while both proMMP2 and active MMP2 were down-modulated (**e,g,i;** p=0.0251; p<0.0001, respectively). The graphs related with transcripts patterns bring the n-fold changes of the profile of transcripts normalized to the β actin, considered here a housekeeping gene.

Mechanotransduction promotes specific epigenetics landscape as potential control of proliferative phenotype and ECM remodeling

Taken our results into account, we suggest a potential effect of shear stress on epigenetic machinery. In order to overall address this issue, the experimental model proposed was newly conducted and the samples were properly harvested to analysis methylation and hydroxymethylation patterns. While 5-meC was unchanged, our date reveals a significant lower global hydroxymethylation (5-hmeC) than the unchallenged cells, as shown at **Fig 12a**, suggesting a potential transcriptional repression, once hydroxymethylation is an intermediate mechanism to demethylation (Richa and Sinha 2014). This data prompted us to evaluate a set of genes encoding epigenetic related enzymes – such as DNMTs (**Fig 12c-e**) and TETs (**Fig 12f-h**). Our data shows that DNMT1 was unchanged as expected, once methylation of cytosine residues is maintained by DNMT1 (Lei et al. 1996) and of all the molecular processes involved in transgenerational epigenetic inheritance, the maintenance of methylation patterns during cell division is the best understood (Chuang et al. 1997; Pradhan et al. 1999).

Additionally, DNMT3A (**Fig 12d**) and DNMT3B (**Fig 12e**) genes were significantly up-modulated, reaching approximately 3-fold changes and 5-fold changes respectively. To note, both of those DNMTs are responsible to *de novo* methylation (Okano et al. 1999), and they seems being a fingerprint of mechanosignaling, maybe in response to ECM remodeling. In fact, epigenetic modifications of chromatin represent a code involved in gene regulation that are clearly responsible for the composition of cell surroundings influencing cell behavior (Vigetti et al. 2014). Also, study reveals the important role of Dnmt1/Dnmt3a in regulating angiogenesis leading to arterial-specific differentiation of hMSCs (R. Zhang et al. 2016). Another point in addition to the signal triggered by ECM remodeling is VEGF, which may play a role upstream of DNMTs through regulating miRNAs or other pathways, which is an interesting study to be addressed.

In addition, TETs 1, 2 and 3 genes were also evaluated, while TET1 was significantly up-expressed (**Fig 12f**), both TETs 2 and 3 were significantly down-expressed (**Figs 12g** and **h**, respectively). Considering the transcriptional profile, it seems clear to suggest that TET1 might explain the global low hydroxymethylation in response to shear stress, once DNA demethylation induces transcription (J. U. Guo et al. 2011), and is regulated by ten-eleven translocation 1–3 (TET1-3), which oxidizes 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine. These intermediates are

then converted to unmodified cytosine by active or passive demethylation mechanisms (Ito et al. 2011; He et al. 2011; Tanaka et al. 2018).

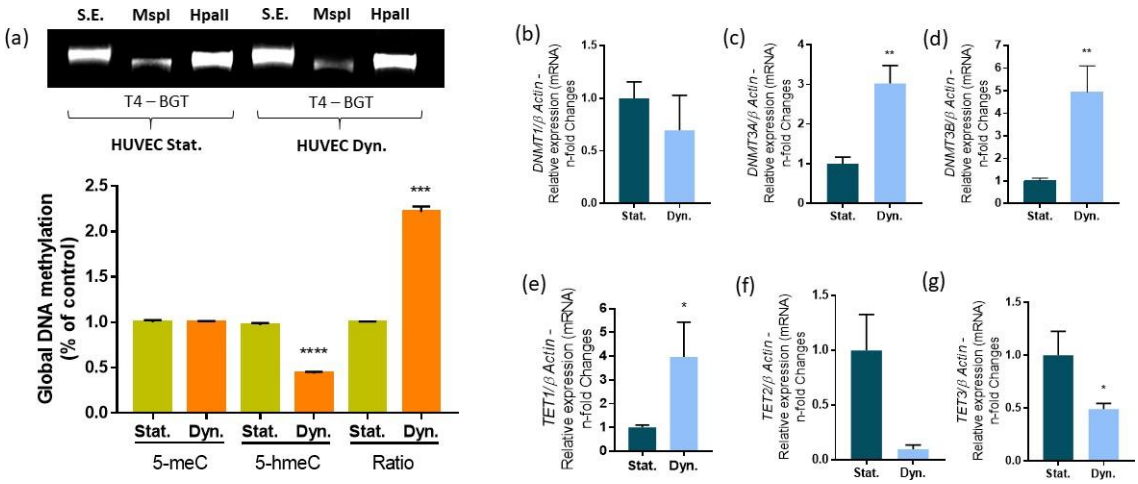

Figura 12: Epigenetic machinery was dynamically modulated in response to shear stress. In order to evaluate the epigenetic landscape, we first considered evaluating the global DNA methylation pattern, processed by T4-BGT DNA glycosylation, followed by MspI and HpaII digestion and agarose gel electrophoresis (**a**). This analysis allows estimate 5-hydroxymethylcytosine and 5-methylcytosine profiles and their densitometries are shown (**b**). The global epigenetic mark is resulted of very dynamic activities of enzymes able to modulate the metabolism of methyl-moiety processing mechanisms of methylation and demethylation, played by DNA methyltransferase (DNMTs) and Ten-eleven translocation (TET) enzymes, respectively. The main genes encoding epigenetic enzymes were as follows: (**c**) DNMT1, (**d**) DNMT3A (p=0.0019), (**e**) DNMT3B (p=0.0043), (**f**) TET1 (p=0.0247), (**g**) TET2 and (**h**) TET3 (p=0.0196).

Taken our results into account it is clear that the mechanosignaling requires a well-controlled ECM remodeling as a pre-requisite to endothelial behavior and this mechanism drives adaptive processes culminating to differential epigenetic marks.

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CAPÍTULO 3

ARTIGO

Laminar shear stress-provoked cytoskeletal changes are mediated by epigenetic reprogramming of TIMP1 in human primary smooth muscle cells

Laminar shear stress-provoked cytoskeletal changes are mediated by epigenetic reprogramming of *TIMP1* in human primary smooth muscle cells

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ABSTRACT

Whereas endothelial responses to shear stress are well-characterized, the cell physiological effects of shear stress in smooth muscle cells (SMCs) remain largely obscure. As SMCs are directly challenged by shear stress after endothelial denuding injury following procedures such as angioplasty or endarterectomy, characterization of these responses represents an important scientific question. Hence we decided to contrast cytoskeletal reorganization, epigenetic reprogramming, signaling transduction and changes in miRNA (miRs) profiles in primary human aortic smooth muscle cells (AoSMCs) between unstressed cells and cells exposed to shear stress. We observed that shear stress provoked reorganization of the actin cytoskeleton in an apparently Cofilin-dependent fashion and which related to altered integrin signaling, apparently caused by remodeling of the extracellular matrix. The latter appeared a downstream effect of increased expression of matrix metalloproteinases and downregulation of tissue metalloproteinase inhibitor 1 (TIMP1) protein levels. In turn, these effects related to shear stress-provoked changes in expression and nuclear localization of the epigenetic regulators demethylases TET1, TET2, DNMT1, DNMT3A and DNMT3B, HDAC6 and SIRT1. Accordingly, *TIMP1* promotor CpG hypomethylation was a prominent effect, and resulted in a significant increase in *TIMP1* transcription which may also have related increased expression of miRs involved in modulating TIMP1 translation. Thus epigenetic-reprogramming of *TIMP1* emerges as critical element in smooth muscle responses to mechanical signals and as epigenetic machinery is amendable to pharmacological manipulation, this pathway may have important clinical consequences.

Key words: Endothelium; Epigenetic; ECM; TIMP; Cytoskeleton; Aortic Smooth Muscle Cells.

INTRODUCTION

Smooth muscle cells modulate blood vessel tone and thus are the principal regulators of blood pressure. Conversely smooth muscle have to adapt to changes in vessel pressure and accordingly smooth muscle cells (SMCs) display remarkable plasticity and can undergo phenotypic changes in response to cues from the local environmental (Pfaltzgraff and Bader 2015). Importantly, while the reaction of the endothelium to mechanical stimulation is well-understood, (Garcia-Cardena and Slegtenhorst 2016) the molecular processes driving SMC adaptation to hemodynamic forces remains largely obscure and it is fair to say that elucidation of these mechanisms remains one of large outstanding questions in vessel biology. Studies report that smooth muscle cells respond to hemodynamic-stress by decreasing survival signaling which may finally culminate in apoptosis and cell loss (Apenberg, Freyberg, and Friedl 2003; Fitzgerald et al. 2008) and there may be important reciprocity between smooth muscle cells and the endothelium, as *e.g* flow-mediated endothelial-derived signals may promote myocyte production of vascular endothelial growth factor-A (Uchida et al., 2015). Clinically, SMCs may be directly exposed to shear stress following endothelial denuding injury as may occur during angioplasty. Studies in restenotic lesions occurring after coronary angioplasty, carotidendarterectomy and vein grafting, suggest that SMCs play a significant role in restenosis (Hao, Gabbiani, and Bochaton-Piallat 2003; Fitzgerald et al. 2008). Thus the understanding the responses of SMCs to shear stress is of substantial clinical and scientific interest.

Generally speaking, cells of the vascular system have to adapt to various mechanical stimuli and thus should be capable of mechanotransduction in order to display adequate cell physiological responses to such stimuli. With regard to mechanotransduction, both the actin-myosin cytoskeleton as well as the extracellular matrix play important roles in resisting and adapting to mechanical forces and both seem to be involved in modulation of gene expression in response to mechanical stimuli (Cho, Irianto, and Discher 2017). Integrins play important roles in the interaction between the extracellular matrix and the cytoskeleton. Accordingly in myofibroblasts from fibrotic lungs, α6-integrin has recently been identified as a mechanosensing integrin subunit and was shown to mediate effects through matrix metalloproteases (Q. Chen et al. 2016). Although the exact molecular mechanisms downstream of integrin signaling-mediated mechanotransduction have not yet been studied directly, the analogy with integrin signaling in general (Abram and Lowell

2009) suggests that this will involve the formation of active signal complex around the cytoplasmic tail in which especially the adaptor protein Cofilin (which amongst other directs the cytoskeletal reorganization following integrin activation) (Willian Fernando Zambuzzi et al. 2009; Bravo-Cordero et al. 2013) and the kinases Src (Meng and Lowell 1998; Willian F Zambuzzi et al. 2008) and focal adhesion kinase (FAK) appear important mediators of the cellular response (Shalaby et al. 1995). As Src and FAK are highly drugable, these kinases may constitute interesting targets for vessel-associated pathological events (Tavora et al. 2014). However, whether also mechanotransduction in vessel smooth muscle cells involves integrin signaling and remodeling of the extracellular matrix and whether conventional integrin signaling mediates the resulting effects remains unknown.

In endothelial cells response to mechanic stimuli prominently involves epigenetic reprogramming of the nucleus, in turn driving alternative gene transcription in these cells. In particular histone deacetylases (enzymes that remove acetyl groups on histone ε-N-acetyl lysine as to wrap the DNA more tightly and inhibit gene expression) have been linked to mechanoresponses in endothelial cells (Lee et al. 2017). Also direct methylation of DNA (which inhibits transcription), through DNA methyltransferases is important in the genomic response to mechanical stimuli of endothelial cells (Dunn, Thabet, and Jo 2015). Other enzymes involved in epigenetic reprogramming, like TET methylcytosine dioxygenases, may be important as well in this respect (Tong et al. 2015). The obvious importance of epigenetic reprogramming in endothelial responses to hemodynamic changes raises questions as to whether similar mechanisms are at work in vessel smooth muscle cells as well. The importance of obtaining answers in this respect is further increased by the presence of clinically useful inhibitors of enzymes involved in epigenetic reprogramming (Prachayasittikul et al. 2017).

The above-mentioned considerations prompted us to explore the biochemical and molecular changes associated with alternative mechanical challenge in human primary aortic smooth muscle cells (AoSMCs). The results show that in AoSMC mechanostimulation provokes hypermethylation of the *TIMP1* promotor among other epigenetic reprogramming events, especially of those linked to increased transcription of matrix metalloproteases. Importantly the resulting decrease in *TIMP1* transcription and translation in conjunction with an increased matrix remodeling enzymatic activity, leads to alternative integrin signaling in vessel smooth muscle cell. In turn the events mediate alternative cellular physiology. Thus these results define a novel-signaling pathway responsible for

physiological adaptation of vessel smooth muscle to changes in mechanical pressure and thus are potentially relevant for defining novel avenues for the rational treatment of disease.

METHODS

1. Reagents and antibodies

Ripa buffer (R0278), Z-Leu-Leu-Leu-al (MG-32, C2211), Phosphatase inhibitor cocktail 2 (P5726), bovine serium albumin (A7906), Fluorosield (F6057), gelatin (48723), saponin (47036), triton X100 (9284) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gotaq qPCR master mix (A6002) was purchased from PROMEGA (Madison, Wisconsin, EUA). DNase I (18068015), High-Capacity cDNA Reverse Transcription and fhalloidin conjugated with Alexa Fluor 488 were purchased from Life Technologies/Molecular Probes, Inc. (Eugene, OR, USA). Antibodies against GAPDH (#2118), β actin (#3700S), Cofilin (#3212), phospho-Cofilin (Ser3) (#3311); Integrin α4 (#4600), PPARγ (#2430S), Erk (4695P), phospho-ERK (Thr202/Tyr204) (#4370P), phospho-GSK3 (Ser9) (#9322), HDAC3 (#9928), HDAC6 (#9928), Sirt 1 (#8499), horseradish peroxidase (HRP)-linked secondary anti-rabbit (#7074) and anti-mouse (#7076) and secondary Alexa Fluor 594 anti-rabbit IgG (#8889) and Alexa Fluor 488 anti-mouse (#4412) IgG conjugated antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-caspase 3 (sc7148), anti-TIMP-1 (sc56489), anti-Ki67 (sc15402) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Fak (ab61113), anti-phospho-β-catenin (Y142) (ab27798), anti-PP2A (ab32141), anti-phospho-PP2A (Y307) (ab32104), anti-Connexin 43 (ab66151) were purchased from Abcam (Cambridge, MA, USA). Anti-DNMT3B (ORB372330), TET1 (ORB228563) and TET2 (orb131790) were purchased from BiorByt (San Francisco, CA, USA).

2.Cell culture

Human primary aortic smooth muscle cells (AoSMC - CC-2571) were purchased from Lonza (Walkersville, MD) and used for experiments between passages 3 to 9. The cells were cultured in SmBM (Smooth Muscle Cell Basal Medium, Lonza, CC-3181) supplemented with SmGM- 2 SingleQuots [0.5 mg/mL hEGF, 5 mg/mL insulin, 1 mg/mL hFGF, 50 mg/mL gentamicin/amphotericin-B, and 5% fetal bovine serum (Lonza, CC-4149)] at 37°C in a

humidified atmosphere containing 5% CO_2. Viability and cell density were determined by the trypan blue dye exclusion test.

3.Shear stress and proteasome inhibition

For shear stress experimentation AoSMCs (10 x 10^4 cells) were seeded in the periphery of modified 100-mm culture dishes sterilized under UV light for 15 min, as described by dela Paz (dela Paz et al. 2012). The cells were seeded in SmBM medium supplemented with SmGM- 2 SingleQuots (LONZA, Walkersville, MD) and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO2. The medium was replaced with DMEM containing 1% of SFB and antobiotics (Nutricel, Campinas, SP) and then confluent monolayers were subjected to orbital shear stress for 72 hours at 37˚C in the presence of CO_2 using an SK-O180-Pro Digital Orbital Shaker (SCILOGEX, Rocky Hill, CT, EUA). For p*roteasome inhibition 5* μM of Z-Leu-Leu-Leu-al (SIGMA, C2211) *was added to* the medium and the cells were submitted to shear stress protocols. A rotation frequency of 100 rpm was chosen. Forces to which cells are exposed can be estimated from the formula: $\tau_{max}=a\sqrt{\rho\eta(2\pi f)^3}$, where ρ = density and η = viscosity and *a* = radius. Considering our experimental condition, $\rho = 937.5\ \frac{kg}{m^3}$, $\eta = 7.5 \cdot 10^4$ *Pa.s* and *a=012m.* Hence our rotation frequency yields stress levels that correspond those observed when considering physiological arterial pressure (6-40 dynes/cm, see Supplementary Fig. S11) (dela Paz et al. 2012). AoSMC from the same passage, which were not subjected to shear stress, were kept in the same CO_2 incubator and were considered the stationary control.

4.Confocal microscopy

For confocal microscopy analysis the cells were grown on glass coverslips placed in the peripheral ring of the culture and submitted to shear stress, as described above. Afterwards, cells were washed with PBS, fixed in PBS-paraformaldehyde (4 % v/v) for 1 h, permeabilized in PBS containing 0.2% Triton-X 100 and 1% BSA at 37°C for 1 h and stained with the appropriate specific primary antibodies, at the concentration recommended by the manufacturer. Then, after washing with PBS to remove the primary antibody, for fluorescence analysis, the cells were stained with Alexa Fluor 594 anti-rabbit or mouse IgG antibody for 1 h. For actin cytoskeleton rearrangement analyses the cells were incubated for 40 min with 4 mg/ml, Alexa Fluor 488-labeled phalloidin (Invitrogen/Molecular Probes,

USA). Cells were washed and coverslips were mounted on glass slides using Fluorosield with DAPI (Sigma) and then viewed on inverted laser scanning confocal microscope (Leica TCS SP5).

5.Picrosirius red stain

For quantitative analysis of collagen content cells were washed with PBS and fixed with paraformaldehyde 4% (v/v) in PBS for 1 h and subsequently stained for 90 min in a 0.1% (w/v) solution of sirius red in saturated aqueous picric acid, pH 2. Cells were then washed for 2 min in 0.01 N HCl, contrast stained with Harris Hematoxylin for 6 min, and followed by rinsing in 70% (v/v) ethanol, dehydrated, cleared, and mounted in Permount (Relatar marca) (Dolber and Spach 1993). For conventional fluorescence microscopy, photomicrographs were taken on the Axio Vert.A1 inverted microscope (ZEISS, Germany) and to determine the collagen content the number of spots in the area recorded in the photo was determined using the ImageJ Software, adjusting the threshold as to properly identify the total collagen and subtypes. For confocal microscopy analysis, the cells were examined using an inverted laser scanning confocal microscope Leica TCS SP5 (LEICA, Germany). The sirius red was excited with a HeNe laser (excitation = 543 nm) and light emission was detected at 560–610 nm and the intensity fluorescence was analyzed using the programs LAS AF (Leica) and images prepared using the Adobe Photoshop CS6.

6.Zymographic analysis

The proteolytic activity of MMP-2 and MMP-9 present in AOSMC-conditioned medium was assayed by gelatin zymography as described by Lefebvre (Lefebvre, Peeters-Joris, and Vaes 1991). Control and shear stressed cultures were used to collect conditioned medium, which was then clarified by centrifugation 13 200 × g for 15 min at 4°C, and stored at −20 °C. Samples were quantified using the Lowry protein assay (LOWRY et al. 1951) and diluted in non-reducing buffer (0.1 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS and 0.001% (w/v) bromophenol blue). Equal amounts of protein (75 µg) were loaded onto SDS–polyacrylamide gel (10% (w/v)) and 4% (w/v) gelatin. MMPs renaturation was performed in 2% (v/v) Triton X-100 for 40 min followed by incubation in incubation buffer [50 mM Tris–HCl and 10 mM $CaCl_2$ (pH 7.4)] at 37 °C for 18 h. Afterwards, gels were stained with 0.5%

(w/v) coomassie blue G 250 for 30 min, washed in a 30% (v/v) methanol and 10% (v/v) glacial acetic acid solution and then analyzed using ImageJ.

7. Western blot

After appropriate treatment AoSMCs were washed in ice-cold PBS and protein extracts were obtained using a RIPA lysis buffer (Sigma Aldrich) supplemented with protease inhibitors (Sigma Aldrich) for 1 h on ice. Protein extracts were cleared by centrifugation 13,200 × g for 15 min at 4°C. Nuclear and cytoplasmic extraction was employed using a hypotonic buffer (20 Mm Tris-HCL, pH7.4, 10 Mm NaCl, 3 Mm $MgCl_2$) and RIPA lysis buffer. The proteins present in the AoSMC cell supernatant were precipitated by the addition of 1 volume of methanol (1:1, v/v) and 0.25 volumes of chloroform (1:0.25, v/v), followed by homogenization and centrifugation at 7,000 × g for 20 min. The upper phase was discarded and 500 µL of methanol was added to the interface. This mixture was centrifuged at 7.000 x g for 20 min and the precipitate containing the proteins was left at 55°C to dry. The precipitate was then resuspended in 100 µl of RIPA lysis buffer (Sigma). The protein concentrations were determined using the Lowry protein assay (LOWRY et al. 1951). An equal volume of 2X SDS gel loading buffer (100mM Tris-HCl, pH 6.8, 200 mM β-Mercaptoethanol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to the samples and boiled for 5 min. Equal amounts of protein (75 µg) were loaded onto SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 1% fat-free dried milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) and incubated overnight at 4°C with appropriate primary antibody at 1:1000 dilution. After washing in TBST, membranes were incubated with appropriate HRP-linked secondary antibodies, at 1:5000 dilutions, in blocking buffer for 1 h. Immunoreactive bands were detected with an enhanced chemiluminescence kit.

8. Analysis of 5-methylcytosine (5-meC) and 5-hydrymethylcytosine (5-hmeC) content

For 5-meC and 5-hmeC *content analysis the g*enomic DNA was initially treated with T4-β-glucosyltransferase (T4-BGT) (New England Biolabs, Beverly, MA, USA), adding glucose moiety to 5-hmC (gDNA) to distinguish amongst DNA methylation and hydroxymethylation. For each sample, three tubes containing 400 ng gDNA each were

treated with 1X NE buffer, 40 mM UDP glucose, T4-BGT (1 unit) to a final volume of 40 µL and incubated at 37°C for 1 h, followed by 10 min at 65°C. Then, samples were digested with *Msp*I or *Hpa*II restriction enzymes (New England BioLabs, Beverly, MA, USA) or H_2O (control) according to the manufacturer's instructions. After digestion, for global DNA methylation analysis 10 µL digestion reactions was electrophoresed on 0.8% agarose gel, stained with SYBR Gold and photographed and then analyzed using software ImageJ. The gene specific analyze, was carried 40 amplification out in a total of 10 µl, containing PowerUp™ SYBR™ Green Master Mix 2X (5 µl) (Applied Biosystems, Foster City, CA), 0.5 µM of each primer, of treated gDNA and nuclease free H_2O. Primers were designed on regulatory regions such as DNaseI hypersensitivity clusters sites, layered by histone modifications marks, CpG regions and transcription factors binding sites, with free primer design and analysis software[30] and further analyzed for secondary structures and annealing temperatures by the Beacon Designer, Free Edition (http://www.premierbiosoft.com/). Sequences and chromosome location were confirmed by the *in-silico* PCR (https://genome.ucsc.edu/). The characteristics of primers and regions of genes analyzed and PCR conditions are illustrated in **Table 2**.

Table 2: Gene methylation-specific primers sequences and PCR cycle conditions.

Gene (ID)	Primer	5'- 3' Sequence	Reactions Condition	Product size (pb)	Restriction ezymes sites
DNMT3B (1789)	**Forward**	**TCAAATTTCCCTCGTCCCCG**	**95°C - 10s; 60°C - 10s; 72°C - 12S**	**129**	**chr 20:** 32.762.285 (site 1) 32.762.322 (site 2) 32.762.343 (site 3) 32.762.349 (site 4)
	Reverse	**GTGCCGACTCCCCTTGTAG**			
TET1 (80312)	**Forward**	**GAGTTGGAAAGTTTGCCCGA**	**95°C - 10s; 57°C - 10s; 72°C - 21S**	**211**	**chr 10:** 68.560.734 (site 1) 68.560.755 (site 2) 68.560.815 (site 3) 68.560.849 (site 4)
	Reverse	**AGAGCCAAGGACGCAGAG**			
TIMP 1 (7076)	**Forward**	**GCCCAGAGAGACACCAGAG**	**95°c - 10s; 59°C - 10s; 72°C - 16S**	**160**	**chr x:** 47.582.487 (site 1) 47.582.506 (site 2)
	Reverse	**CTAGACTAGCCCAGGGTCCT**			

9.Gene expression analysis

Quantitative polymerase chain reaction (qPCR) was performed using a *QuantStudio® 3* Real-Time PCR System to assess changes in mRNA expression in the following genes reported in **Table 3**. Total RNA was extracted from cells with Ambion TRIzol Reagent (Life Sciences - Fisher Scientific Inc, Walthan, MA, USA) and treated with DNase I (Invitrogen, Carlsband, CA, USA). cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. qPCR was carried out in a total of 10 µl, containing PowerUp™ SYBR™ Green Master Mix 2X (5 µl) (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, 50 ng of cDNA and nuclease free H_2O. Fold changes were analyzed using the comparative CT method (ΔΔCt) normalizing to GAPDH expression and compared to static conditions as a reference.

Table 3: Expression primers sequences and PCR cycle conditions.

Gene (ID)	Primer	5'- 3' Sequence	Reaction Condition
MMP9 (4318)	Forward Reverse	CAC GCA CGA CGT CTT CCA AAG CGG TCC TGG CAG AAA T	95°C - 15s; 60°C - 30s; 72°C - 30s
MMP2 (4313)	Forward Reverse	AGC TCC CGG AAA AGA TTG ATG CAG GGT GCT GGC TGA GTA GAT	95°C - 15s; 60°C - 30s; 72°C - 30s
TIMP1 (7076)	Forward Reverse	CCG CAG CGA GGA GTT TCT C GAG CTA AGC TCA GGC TGT TCC A	95°C - 15s; 60°C - 30s; 72°C - 30s
DNMT1 (1786)	Forward Reverse	AGG ACC CAG ACA GAG AAG CA GTA CGG GAA TGC TGA GTG GT	95°C - 15s; 60°C - 30s; 72°C - 30s
DNMT3A (1788)	Forward Reverse	AGG AAG CCC ATC CGG GTG CTA AGC GGT CCA CTT GGA TGC CC	95°C - 15s; 60°C - 30s; 72°C - 30s
DNMT3B (1789)	Forward Reverse	TCG ACT TGG TGG TTA TTG TCT G TCG AGC TAC AAG ACT GCT TGG	95°C - 15s; 60°C - 30s; 72°C - 30s
TET1 (80312)	Forward Reverse	GCC CCT CTT CAT TAC CAA GTC CGC CAG TTG CTT ATC AAA ATC	95°C -15s; 60°C - 30s; 72°C - 30s
TET2 (54790)	Forward Reverse	GGT GCC TCT GGA GTG ACT GT GGA AAA TGC AAG CCC TAT GA	95°C -15s; 60°C - 30s; 72°C - 30s
SIRT 1 (23411)	Forward Reverse	GGT CAG GCT GGT TTA CAA CG GGC ATA GAC CCA CAC ACA TCT	95°C -15s; 60°C - 30s; 72°C - 30s
Actin	Forward Reverse	AGA GAG CCT CGC CTT TGC GCG GCG ATA TCA TCA TCC	95°C -15s; 60°C - 30s; 72°C - 30s

10.Pri/pre microRNAs expression analysis

For pre- and pri-micro RNAs, both sense and antisense primers were designed to be located within the hairpin sequence of the miRNA precursors. The sequences of pre-miRNAs are downloaded from miRBase (http://www.mirbase.org/) and primers set designed by program OligoAnalyzer 3.1 (https://www.idtdna.com/) in to the hairpin should simultaneously amplify both RNAs. Real-time quantitative PCR was performed a *QuantStudio® 3* Real-Time PCR System according to the manufacturer's protocol (Promega, Madosin, USA). Briefly, 10 µL reaction contained 100 ng reverse transcription products, 500 nM of each primer, 5 µL SYBR Green PCR master mix, 0.2 µL ROX, and 3 µL nuclease free H_2O. The reactions were prepared in a 96-well plate, and the thermal cycler program was as follows: at 95°C for 15 min, followed by 40 cycles at 95°C - 15 s, 60°C - 30 s and then 72°C - 30 s on ABI 7500 Real-Time PCR System. The reactions were run in triplicate and included no template control for each gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (Ct) was determined for each gene, and the relative amount of each miRNA to miR-17 was described using the equation $2^{-\Delta Ct}$ where ΔCt - (Ct^{miRNA} - Ct^{miR17}) (Tong et al., 2015) and Fold changes were analyzed using the comparative CT method ($\Delta\Delta Ct$). All the sequences of the microRNA and primers and PCR conditions are listed in **Table 4**.

Table 4: Pri/pre microRNAs expression primers sequences and PCR cycle conditions.

microRNA (accession number)	Primer	5'- 3' Sequence	Reactions Condition
hsa-mir-365[a] (MI0000767)	Forward	CCG CAG GGA AAA TGA GGG ACT TTT G	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGC AAG AGC AAT AAG GAT TTT TAG GGG CAT TAT G	
hsa-mir-22 (MI0000078)	Forward	GGC TGA GCC GCA GTA GTT CTT C	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GCA GAG GGC AAC AGT TCT TCA ACT G	
hsa-mir-143 (MI0000459)	Forward	CGC AGC GCC CTG TCT C	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GCT GCA GAA CAA CTT CTC TCT TCC TGA G	
hsa-mir-145 (MI0000461)	Forward	ACC TTG TCC TCA CGG TCC AGT TTT C	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	AAC CAT GAC CTC AAG AAC AGT ATT TCC AGG	
hsa-mir-10b (MI0000267)	Forward	CCA GAG GTT GTA ACG TTG TCT ATA TAT ACC CTG TAG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGA AGT TTT TGC ATC GAC CAT ATA TTC CCC TAG	
hsa-mir-16-1 (MI0000070)	Forward	AGC AGT GCC TTA GCA GCA CG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GTC AAC CTT ACT TCA GCA GCA CAG TTA ATA C	
hsa-mir-17 (MI0000071)	Forward	TCA GAA TAA TGT CAA AGT GCT TAC AGT GCA GG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GTC ACC ATA ATG CTA CAA GTG CCT TCA CTG	
hsa-mir-21 (MI0000077)	Forward	GTC GGG TAG CTT ATC AGA CTG ATG TTG AC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TTG TCA GAC AGC CCA TCG ACT GG	
hsa-mir-1293 (MI0006355)	Forward	AGG TTG TTC TGG GTG GTC TGG AG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TAA AGA CTA AGT GGT CCG GAG ATT TGT GC	

11.Statistical analysis

All experiments were performed at least three times. Results were expressed as mean ± standard deviation. Statistical analysis was performed by Student's t test, or by analysis of variance (ANOVA) followed by the post hoc Tukey test when more than two groups were compared, using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, EUA). Differences were considered significant at $P < 0.05$ in two-sided tests of statistical significance. Densitometric analyses of blots and zymograms were performed using Scion Image software.

RESULTS

In vitro exposure to shear stress provokes physiological adaptation in AoSMCs

The systolic/diastolic cycle to which vessel cells are subjected is well known to cause physiological adaptation of these cells and aberrations in this adaption are linked to development of various pathological conditions (Huveneers, Daemen, and Hordijk 2015). Mechanosignaling in endothelial cells is now fairly well understood, (Green et al. 2017) the mechanisms operative in vascular smooth muscle remain, however, largely obscure. Hence, we decided to investigate whether it is possible to model physiological adaptation to mechanical forces in human primary AoSMCs. To this end, AoSMCs cultures were either left stationary or subjected to mechanical stimulation (**Fig.13**). After 72 hours the AoSMCs cultures involved were fixed and subjected to investigation using confocal microscopy.

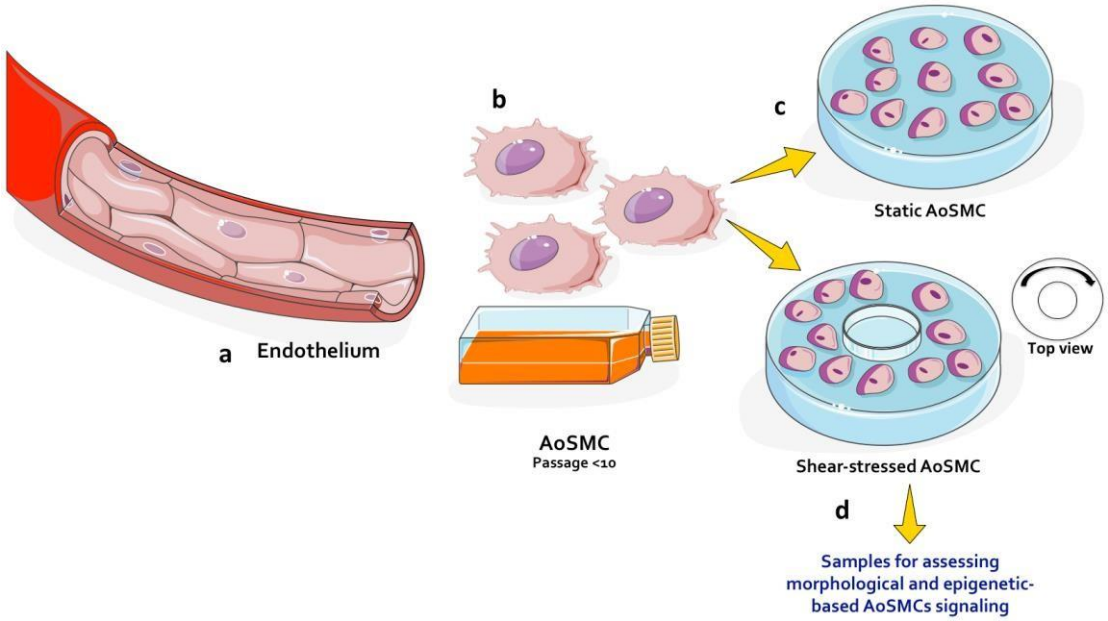

Figure 13: Summary of the experimental procedures and the associated timeline. **a.** Schematic representation of endothelium formed by distinct endothelial and smooth muscle cells cross-talking with each other, in the context of a Extracellular-matrix (ECM). Both cell lineages are subject to mechanotransduction in response to systolic/diastolic arterial blood pressure changes (represented by the arrows); **b.** Primary Aortic Smooth Muscle Cells (AoSMCs) were purchased from LONZA and maintained into incubator at classical conditions up to passage 10 as recommended by the manufacturer; AoSMCs were expanded and later split in two groups: shear stress-exposed cells and static controls; **c.** Shear stress was provoked by using a incubator-adapted shaker (6-40 dynes/cm), while in the static control group the cells remained in the same condition by without being submitted to shaking; **d.** After 72 hours, the cells were probed by confocal microscopy and biochemical approaches, also in order to identify epigenetic changes. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

Study of the actin cytoskeleton using phalloidin revealed substantial reorganization following shear stress stimulation, in addition to a clear increase in F-actin polymerization. This actin polymerization was evidently vector tangential to the direction of the shear stress (**Fig.14a**). Also Cofilin and FAK, two other proteins canonically associated with mechanoperception, (Eitenmuller et al. 2006; Wu et al. 2007) reorganized according to the flow applied to the smooth muscle cells: (**Fig.14a-i,** Supplementary **Fig.S1**). The specificity of these effects was demonstrated by the observation that subcellular distribution of Integrin-α4 (Supplementary **Fig.S2**) was not affected. Furthermore, Western blot analysis contrasting control cultures to shear-stressed AoSMC showed that mechanical load decreases the ratio of phospho-Ser3-Cofilin over *pan*-Cofilin (**Fig.14i;** Supplementary **Fig.S3**). The latter effect may involve PP2A (**Fig.15e** and **f;** Supplementary **Fig.S4**). As phospho-Ser3-Cofilin represents the inactive form of the protein, (Wang, Eddy, and Condeelis 2007) these results further confirm physiological adaptation of AoSMC to mechanical stimuli.

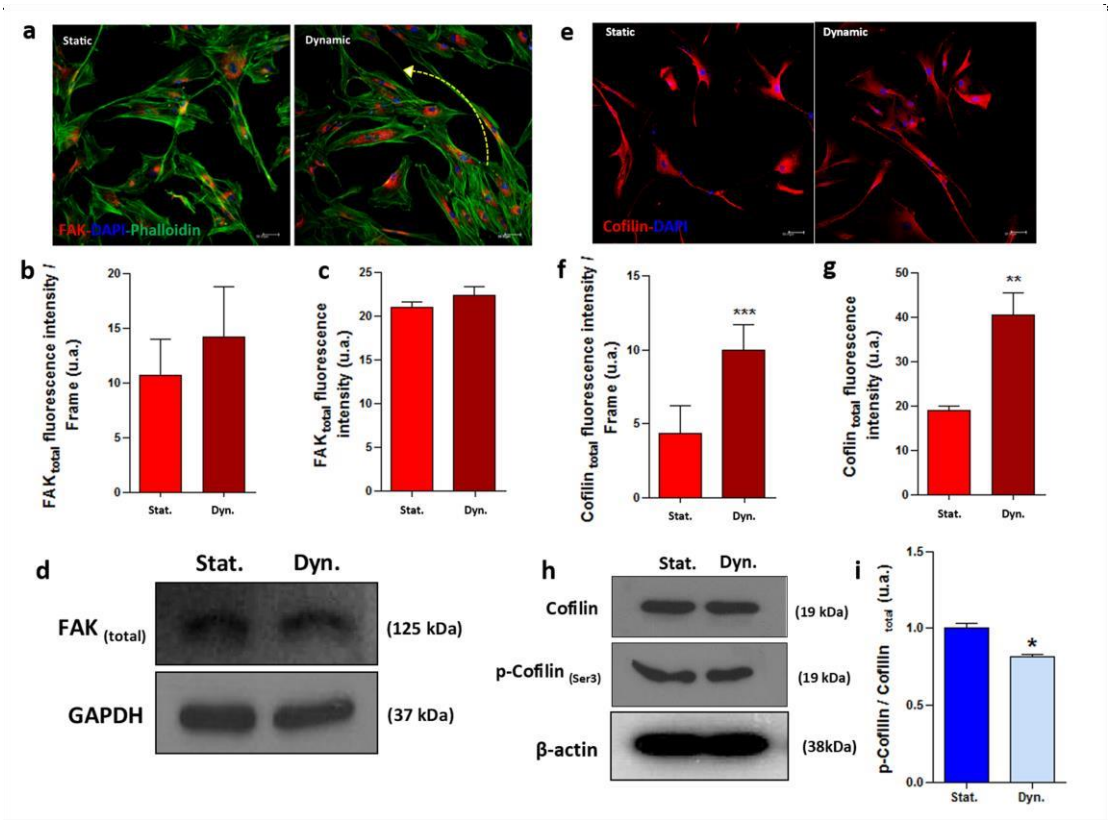

Figura 14: Cytoskeleton dynamics depend on FAK/Cofilin signaling to provoke AoSMC phenotypic changes. Mechanically-stressed AoSMC and control cultures were investigated for FAK and Cofilin subcellular distribution as well as F-actin organization were evaluated by laser confocal microscopy employing specific antibody for FAK (**a**) and Cofilin (**e**), followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and Alexa Fluor 488-conjugated phalloidin (green) (**a**). The nuclei were stained with DAPI (blue). The total FAK (**b** and **c**) and Cofilin (**f** and **g**) intensity fluorescence was analysed using the program LAS AF. For WB analyses equal amounts (75 µg) were loaded per lane and blotted with specific antibodies. One representative immunoblot of total FAK (**d**), Cofilin or phospho-cofilin (**h**) and phospho-cofilin/cofilin ratios (**i**) is presented. Densitometric analysis of immunoblots was normalized to the protein ratio of controls (1) and GAPDH was used as loading control. Images are representative of three independent experiments. Bar = 50 µm. Results were represented as mean ± standard deviation of three independent experiments. *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ compared with Static. Bar = 50 µm. Representative results of 3 independent experiments.

Finally, a conventional response to hemodynamic stimulation in endothelial cells is the activation of the Wnt/ß-catenin pathway, (McCue et al. 2006; Gelfand et al. 2011). As judged by the increased dephosphorylation of phospho-Ser9-Glycogen Synthase Kinase 3ß (**Fig.15a** and **b**) and the increased nuclear translocation of ß-Catenin (**Fig.15j** and **l**), this was a prominent response to shear stress-challenged AoSMC cultures as well. Additionally, we also observed that mechanically stressed AoSMCs are characterized by relative upregulation of survival signaling. Increased phosphorolyation of p42/p44MAPK/ERK were seen, and correspondingly Ki67 (a cell cycle progression biomarker) staining increased (Supplementary **Fig.S5**), while simultaneously caspase-3 remained unchanged (Supplementary **Fig.S5**) between the different experimental conditions. Connexin43 expression was significantly decreased in the group exposed to mechanical stress, however its localization in the cell appeared overy similar under the different conditions (Supplementary **Fig.S6**).

We concluded that our experimental set up allows investigation of the molecular mechanisms involved in physiological adaptation of vascular smooth muscle cells to mechanical force and that adaption to such stimuli in vessel smooth muscle cells is *grosso modo* similar to that seen in the endothelial compartment. Subsequently experiments were initiated to identify the mechanisms mediating adaptation to mechanical stimulation.

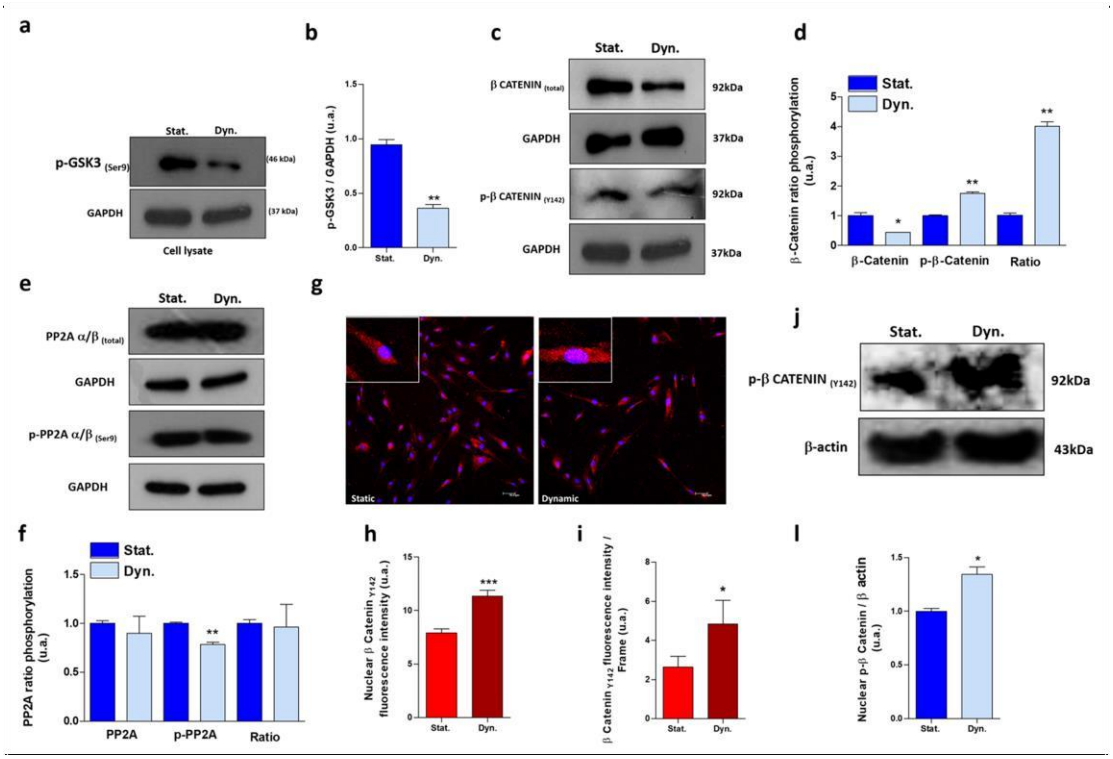

Figure 15: Mechanical stress activates GSK3ß-mediated ß-Catenin signaling in AoSMCs. For the analysis of the levels or phosphorylation status of endogenous WNT protein, equal amounts (75 µg) were loaded per lane and investigated on Western blot with specific antibodies. A representative immunoblot of total cell lysates (**a**) and nuclear extract (**j**) are shown. Results of densitometric analysis of immunoblots are expressed as the relative intensity ratio of phospho-β Catenin (**c** and **d**), phospho-GSK3 (**a** and **b**), phospho-PP2A/PP2A (**e** and **f**) and nuclear phospho-β Catenin (**j** and **l**) by β-actin or GAPDH, normalized to the protein ratio of controls (1). For probing β Catenin subcellular location, by laser confocal microscopy was employed (**g-i**) Results of staining cultures employing a specific antibody for phospho-β Catenin, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red). The nuclei were stained with DAPI (blue). The amounts of total phospho-β Catenin (**f**) and nuclear (**g**) intensity fluorescence was analyzed using the programs LAS AF. Images are representative of three independent experiments. Bar = 50 µm. Results are represented as mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.001 and ***p < 0.0001 compared with the static condition.

Physiological adaptation in response to flow stimulation results from cell-autonomous altered expression of MMPs / TIMP1 and subsequent extracellular matrix (ECM) remodeling

It is well known that cells can react to mechanical forces through alterations of the extracellular environment, which are then subsequently sensed by the cells involved and in turn this provokes remodeling of the F-actin cytoskeleton (Sun, Guo, and Fässler 2016). The results described above suggest that similar mechanisms might be operative in our experimental system. Thus we decided whether vascular smooth muscle cells cell-autonomously react to flow by altering the ECM. To this end we studied expression and activity of MMPs and their inhibitors. As shown (**Fig.16**), AoSMCs display strong down-regulation of both TIMP1 translation and secretion upon mechanical challenge using a variety of technical approaches (**Fig.16a-g**). Functionally this correlated to a decrease in collagen deposition in flow-stimulated AoSMCs cultures as assessed by picrosirrus dye staining (Supplementary **Fig. S7**). A detailed analysis established that this decrease in total collagen content stems from lower amounts of collagen I, III and IV, but paradoxically collagen II is increased following mechanically stress (**Fig.16h** and **i**; Supplementary **Fig. S7**). Concomitantly, we also investigated MMPs and we observed a significant increase in MMP2 transcription (**Fig.4j** and **k),** while MMP9 is not affected by flow stimulation (**Fig.16k)**. AoSMCs conditioned medium was further probed by SDS-PAGE-based zymography and results corroborated the notion that MMP2 activity (**Fig.16l** and **m)** is increased in mechanically-stressed AoSMCs cultures. We thus concluded that mechanical challenge provokes cell-autonomous remodeling of the ECM through decreasing TIMP1 and increasing MMP2 enzymatic activity, which in turn directs the physiological adaptation of smooth muscle cells to such stress.

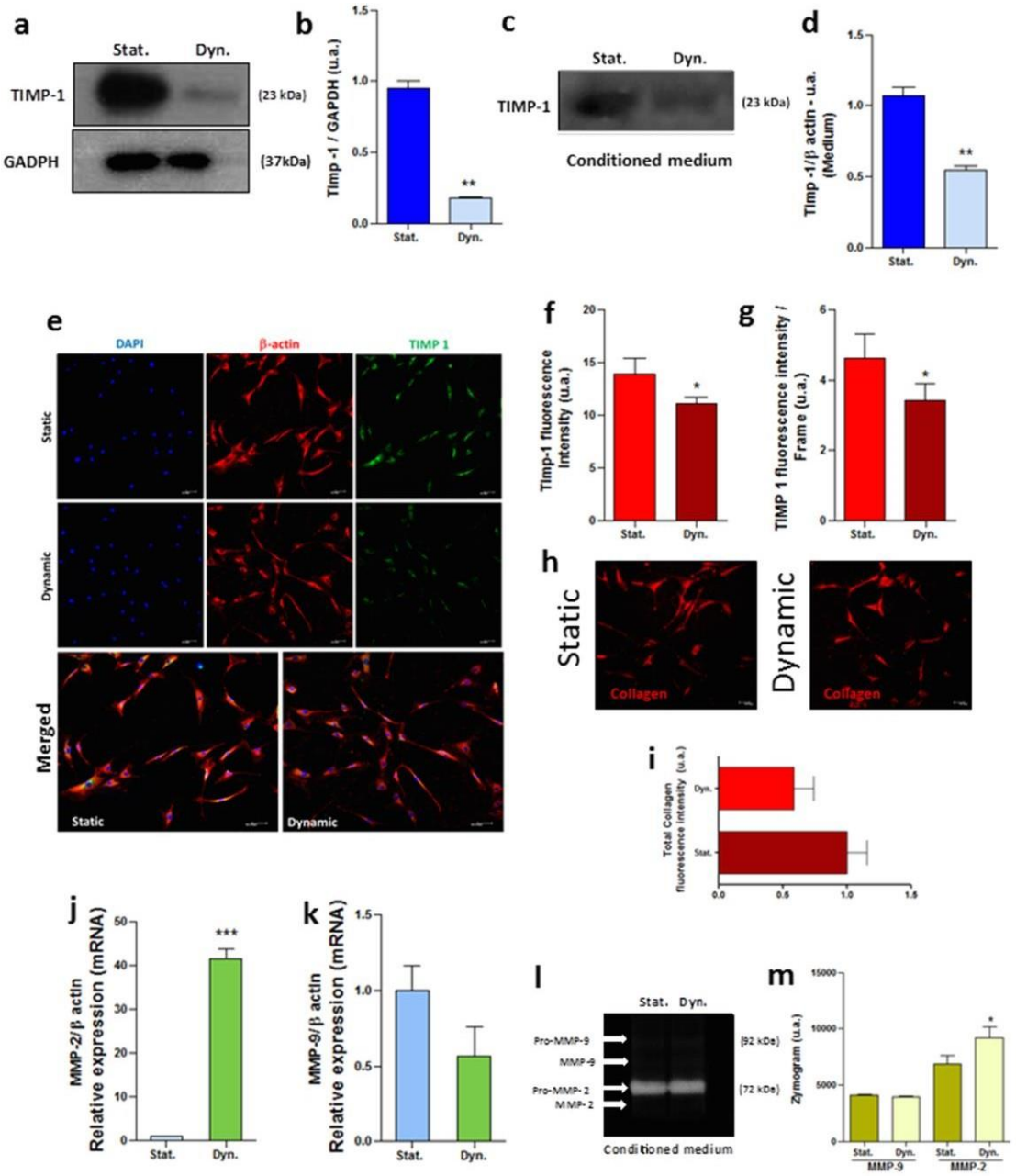

Figure 16: MMPs and TIMP1 and ECM-remodeling in response to shear-stress. Comparison of mechanically challenges and static control cultures for the levels endogenous of tissue inhibitor of metalloproteinase (TIMP-1) as analyzed by Western blots of cell lysates (**a**) and conditioned medium (**c**). Densitometric analysis of immunoblots involved is expressed as the relative intensity of TIMP-1/β-actin or GAPDH ratio normalized to the protein ratio of controls (**b** and **d**). TIMP-1 SUBCellular location and total culture collagen content were evaluated by laser confocal microscopy after incubation of the cells with specific antibody for TIMP-1, followed by staining with Alexa Fluor 488 goat anti-rabbit IgG antibody (green) or picrosirius red *stain (**h**). Results of* staining employing an antibody for β-actin, followed by Alexa Fluor 594 goat anti-mouse IgG antibody (red) and nuclei stained with DAPI (blue). Bar = 50 µm. *TIMP-1 (**f** and **g**) and total Collagen (**i**) fluorescence intensity* was analyzed using LAS AF software. MMP-2 (**j**) and MMP-9 (**k**) gene expression was detected by qPCR analysis

normalized by β-actin gene expression level and activity by zymographic analysis in conditioned medium (**l** and **m**). Immunoblots, images and zymogram are representative of three independent experiments. Results are represented as mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.001 and ***p < 0.0001 compared to the static condition.

Epigenetic reprogramming mediates responses to mechanical challenge in primary human AoSMCs

Recently it has become clear that epigenetic mechanisms are intimately involved in the reaction to mechanical stimulation in the endothelial compartment (Lee et al. 2017) and hence such mechanisms may well be implicated in the responses to flow in our experimental system as well. A first indication that epigenetic mechanisms are important in the context of mechanically challenged AoSMCs as well, was obtained in experiments in which we investigated the overall content of cultures for 5meC and 5hmeC. While mechanostimulation down-regulated 5meC levels; 5hmeC was upregulated in mechanically-stressed AoSMCs (**Fig.17a** and **b)**.

This observation encouraged us to investigate important regulators of the balance between methyl and acetyl moieties, including DNMTs, TETs, $HDAC_3$, HDAC6 and SIRT1. Indeed, additional proof for an involvement of epigenetic mechanisms in AoSMC responses to mechanostimulation came from qPCR experiments that showed that treatment upregulated mRNA levels of DNMT1 (**Fig. 17c),** DNMT3a (**Fig. 17d)** and $DNMT_3b$ (**Fig. 17e),** while conversely both TET1 (**Fig. 17f)** and TET2 (**Fig. 17g)** were significantly down-regulated**.** These results were also evident at the protein level, at least for $DNMT_3B$ (**Fig. 17h** and **i),** TET1 and TET2 (**Fig. 17h,j,k),** although effects on nuclear levels of TET1 proteins were, although statically significant somewhat marginal raising questions as to the functional importance of this response (**Fig. 17z, z1,** z2**)**. Additionally, the promotors of the both *TET1* and *DNMT3B* genes were modulated by epigenetic mechanisms since both displayed differentially levels of methylated (5meC) and hydroxymethylated (5 hmeC) residues (**Fig. 17w** and **y**). Furthermore, mechanostimulation upregulated mRNA levels of $HDAC_3$ and SIRT1 (**Fig. 17l-n**) and down-regulated $HDAC_3$ protein content, and both SIRT1 and HDAC6 were significantly upregulated (**Fig. 17o-r)**. Further indication that the changes observed are functional came from experiments in which we studied the subcellular distribution of SIRT1 (**Fig. 17s-u**) and showed

an increase of nuclear translocation of this enzyme following mechanostimulation (**Fig. 17v,x;** Supplementary **Fig.S8)**.

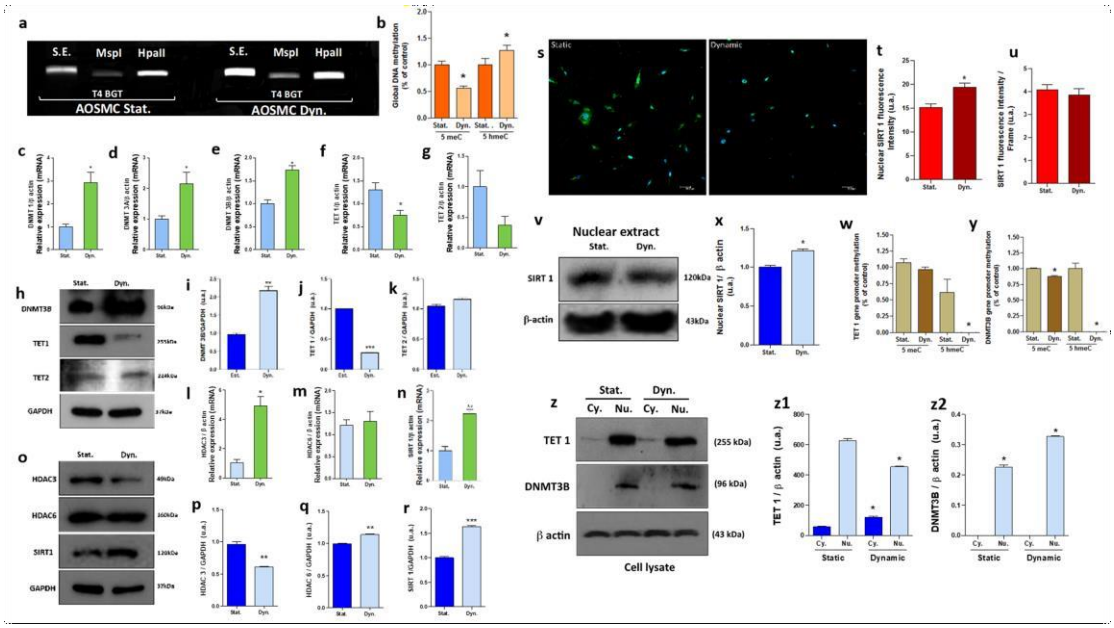

Figure 17: Epigenetic organization of AoSMCs is influenced by mechanical stress. The global (**a**) DNA methylation pattern of AoSMC cultures (either exposed to shear stress or that of static controls) was analyzed by DNA glucosylation with T4-BGT, followed by MspI and HpaII digestion and *agarose* gel *electrophoresis for global DNA methylation and* real time PCR of promoter sequences. The results show the densitometric analysis of global 5-hydroxymethylcytosine and 5-methylcytosine levels (**b**). Transcript levels of epigenetic reorganization-related genes were investigated (**c-g**). Translate levels of epigenetic reorganization-related genes were investigated by immunoblot (**h,o**). HDAC 3 (**o,p**), HDAC 6 (**o,q**) and SIRT1 (**o,r**) were investigated. SIRT1 nuclear levels were investigated by immunofluorescence and LAS AF software (**s-u**). Validation by immunoblotting of nuclear extracts (**v,x**). DNMT3B (**h,i**), TET1 (**h,j**) and TET2 (**h,k**) levels investigated by immunoblotting and nuclear translocation (**z**). TET1 and DNMT3B gene promoter methylation (**w,y,** respectively). Densitometric immunoblotting analysis is expressed as the relative intensity of proteins/GAPDH or β-actin ratio normalized to the protein ratio of controls**.** Genomic DNA electrophoresis gel**,** immunoblots and images are representative of three independent experiments. Bar = 50 µm. Results are represented as mean ± standard deviation of three independent experiments. *p < 0.05**,** **p < 0.001 and ***p < 0.0001 compared to the static condition.

In conclusion**,** exposure of AoSMCs to mechanical stimulation substantially alters the epigenetic controlling landscape in these cells. In conjunction with existing body of data on the activation of such mechanisms by blood pressure in endothelial cells (Dunn**,** Thabet**,** and Jo 2015) these data suggest that such responses can be considered a canonical element in vessel cell responses to changes in mechanic stimulation.

Vascular smooth muscle cell TIMP1 protein downregulation involves changes in the epigenetic control of this gene.

In an effort to link the effects observed on the epigenetic landscape of AoSMCs to the physiological responses to mechanostimulation of these cells, we contrasted the methylation profile of the *TIMP1* gene promoter in static and dynamic AoSMCs cultured. Under dynamic conditions there is significant hypomethylation with respect to both 5meC and 5hmeC (**Fig.18a),** providing a rational explanation for the observed differences in AoSMC *TIMP1* transcripts (**Fig. 18b)**. However, although *TIMP1* was epigenetically reprogrammed in order to modulate *TIMP1* transcription, the amount of TIMP1 (protein) was significantly decreased. Potential explanations include miRNA-mediated alternative posttranscriptional processing or protein destruction by a proteasome-dependent pathway. With respect to the latter, there is no major involvement of the proteasome, since by treating AoSMCs with MG32, a classical proteasome inhibitor, did not counter effects of flow on TIMP1 protein levels (Supplementary **Fig.S9**).

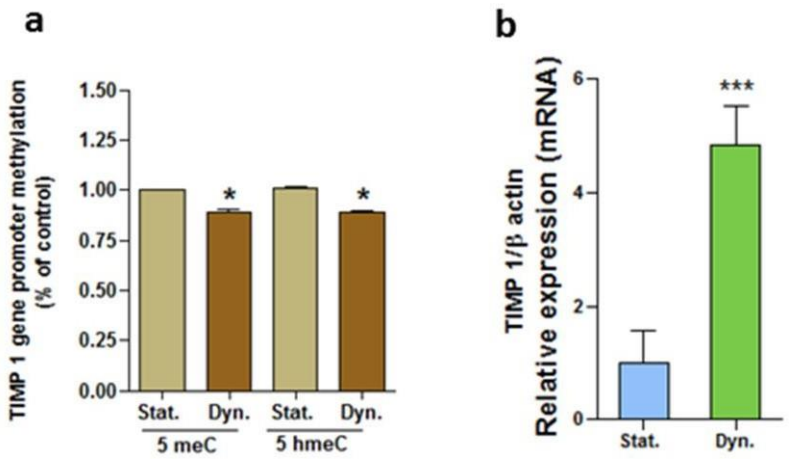

Figure 18: Epigenetic regulation of TIMP 1 expression through promoter methylation. AoSMC cultures were exposed to shear stress or remained under static conditions and methylation of TIMP 1 gene promoter was investigated (**a**) as were transcript *levels* by qPCR (**b**). The apparent discrepancy with TIMP1 protein levels (presented elsewhere in this manuscript) suggest important roles for posttranscriptional mechanisms explaining effects of mechanical challenges on TIMP1 amounts.

However, an analysis for the requirement of TIMP1 processing-related miRs (P. Li et al. 2013) by qPCR (Tong et al. 2015), suggested involvement of miRs in this response to shear-stress. In total, we evaluated 9 miRs and in response to mechanical forces levels miR1293, miR23a, miR143, miR365 and miR 145 were significantly increased (**Fig.19a-e)**. In addition there was trend to upregulation of miR10b, miR22, miR16 and miR 21 as well, but these effects did not reach statistical significance (Supplementary **Fig.S10**). Importantly, miR365 (**Fig.19e**, which reached a 20 times fold change in response to shear stress) has previously been identified as a mechanosensitive microRNA targeting histone deacetylase 4, (Guan et al. 2011). Additionally, mechanostimulation upregulated mRNA levels of DROSHA and XPO5 (**Fig. 19f** and **g),** while DICER remained without any significance (**Fig. 19h)**. To date, these molecules are important members of miRs processing machinery.

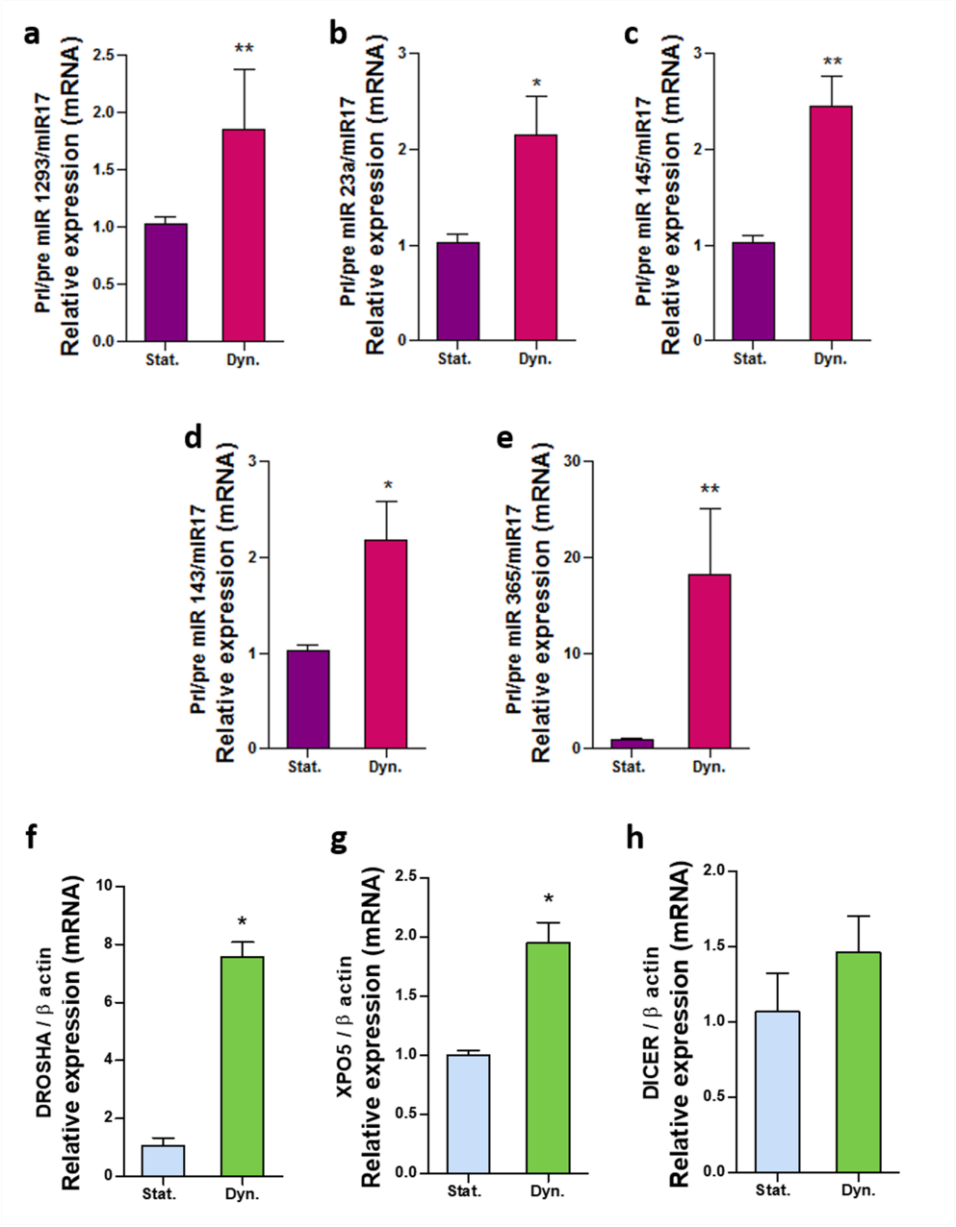

Figura 19: microRNA processing guarantees TIMP1 protein downregulation. Pri/pre miRNAs [miR1293 (**a**), miR23a (**b**), miR145 (**c**), miR143 (**d**) and miR365 9 (**e**)] were detected by qPCR and results were normalized to miR17 expression (considered to have constitutively constant levels of expression as suggested by Peltier and Latham, 2008). DROSHA, XPO5 and DICER genes were also evaluated (**f-h**, respectively). Results are represented as mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.001 compared to the statically-maintained control cultures.

Thus, a picture emerges in which human vascular smooth muscle cells react to vessel pressure by profound reorganization of the epigenetic landscape manifesting itself by altered expression of enzymes controlling ECM composition. The resulting changes in the extracellular environment then mediate morphological adaptation to mechanical stress in this cell type (**Fig.20**).

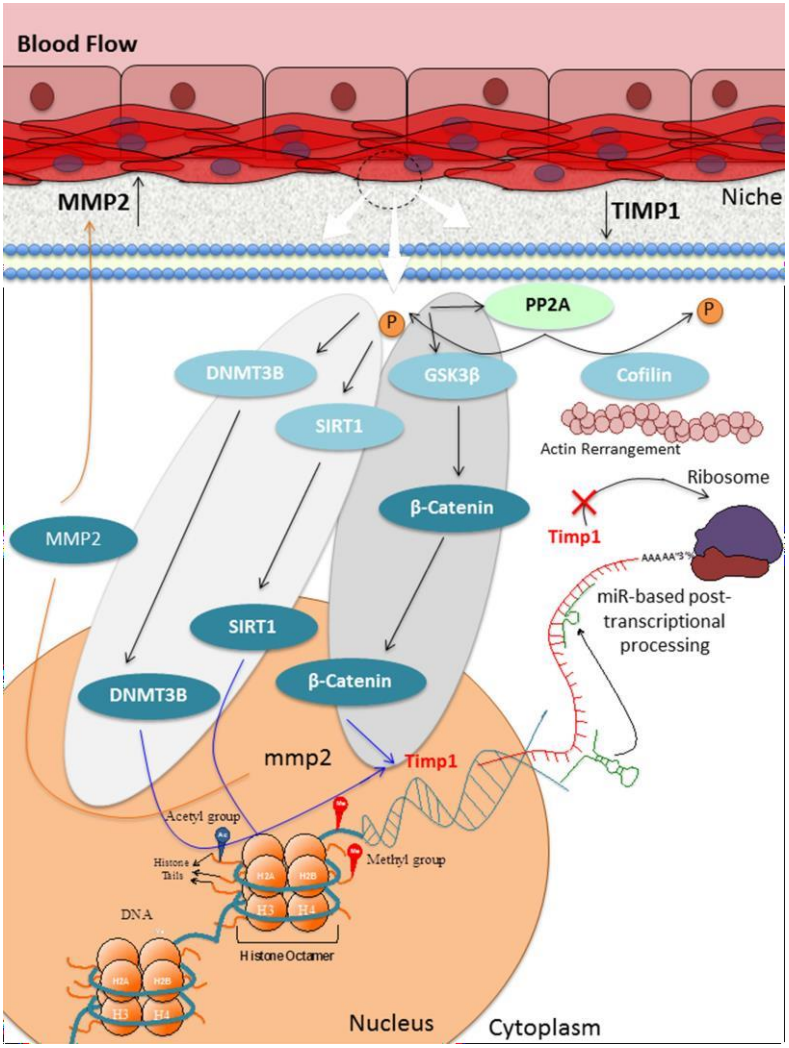

Figure 20: Schematic representation of the molecular effects of shear stress in primary human AoSMC cells. Here we set to contrast primary cultures of human aortic smooth muscle cells under static and flow dynamic conditions. The results show that flow mechanical challenge of such cultures provokes morphological and physiological adaptation in smooth muscle cells through remodeling of the ECM, which in turn is mediated by changed expression of matrix remodeling controlling enzymes through a profound reorganization of the epigenetic landscape, a particular important role emerging for altered control of TIMP1. Thus our results define a novel pathway mediation effects mechanical stimulation in the vascular smooth muscle compartment.

DISCUSSION

It is well established that cells of the blood vessels adapt themselves to changes in blood pressure. For endothelial cells (Shah et al. 2017) and also pericytes (Hall et al. 2014) the molecular mechanisms involved are now fairly well understood. However, how large artery smooth muscle adapts to hemodynamic forces is much less resolved. Although SMCs are usually not directly exposed to flow, procedures like angioplasty can denude such cells from the protective endothelial layer, leading abrupt laminar shear stress. To date, restenosis after vascular intervention is a major source of interventional failure leading to mortality and morbidity in patients.

Here, we set out to contrast primary cultures of human aortic smooth muscle cells under static and flow dynamic conditions. The results show that flow mechanical challenge of such cultures provokes morphological and physiological adaptation in smooth muscle cells through remodeling of the ECM, which in turn is mediated by changed expression of matrix remodeling controlling enzymes through a profound reorganization of the epigenetic landscape (**Fig.8**), with a particular important role emerging for altered control of *TIMP1*. Thus our results define a novel pathway mediating effects mechanical stimulation in the vascular smooth muscle compartment.

A role for ECM remodeling for physiological adaptation of vascular smooth muscle cells to stress responses fits wells with established mechanisms in the endothelial compartment (Shi et al. 2016). We observed that expression of both MMPs and their regulators is substantially altered in the smooth muscle compartment. In view of the notion that the size of smooth muscle compartment is substantially larger in vessels as that of the endothelial compartment, it is tempting to speculate that ECM remodeling in response to changes in pressure mostly relates to responses in smooth muscle. In this sense the smooth muscle compartment might be primarily responsible for translating mechanical stress to altered ECM composition and should thus be considered as the main driving force in the physiological response to altered pressure and it should prove interesting to investigate this notion directly, employing *e.g.* experimental systems with a selective genetic ablation of *TIMP1* in the smooth muscle compartment while leaving expression in the endothelium unaffected.

Mechanisms operative in smooth muscle appear to reflect, at least to some degree, those described earlier in endothelium and pericytes. Effects on seen with respect to the

regulation of TIMP1 clearly echo those seen earlier by Uchidan and Haas in endothelial cells. (Uchidan and Haas, 2014). This may relate to the idea that such cells can be ontogenetically related (Y.-X. Chen et al. 2017). Epigenetic responses to flow stimulation are well-documented in endothelial cells (Khyzha et al. 2017) and are similar to those seen in the present study in AoSMCs. Likewise, we observed modulation of Wnt signal transduction in mechanically-challenged AoSMCs and such observations have also been done in endothelial cells and even been linked to altered TIMP-1 expression. Thus our results point to the existence of a general flow-responsive pathway in vessel cells, involving sequential epigenetic reprogramming, ECM modulation and physiological adaptation, in which only limited cell type specificity appears present. Further experiments in which static and dynamically-challenged endothelial cultures are compared head-on to smooth muscle culture could be instrumental in further substantiating this notion.

An important limitation of the present study is that only one type of vascular smooth muscle, *in casu* derived from human aorta was tested. To further test the general applicability of the findings described above, it may prove imperative to also repeat the experiments described in other types of smooth muscle. In general, although for pericytes and endothelial cells important roles of regionality have been described, there is little evidence that is an important issue for vascular smooth muscle cells (Molema 2010; Nikolakopoulou et al. 2017). Nevertheless, until studies with other types of smooth muscle have been performed, certain carefulness with extrapolating the results of present study to the vascular bed in general should be exercised.

Disregarding the above-described caveats, however, the present study has revealed the existence of a complex but important pathway mediating physiological responses to mechanical stimulation operative in smooth muscle cells. This pathway involves altered epigenetic control of ECM modulating enzymes following exposure to blood flow. As the epigenetic machinery is amendable to pharmacological manipulation and as especially the smooth muscle layer is important in atherosclerosis elucidation and others, this pathway may have important clinical consequences.

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Conflict of interest

The authors declare no competing financial interests.

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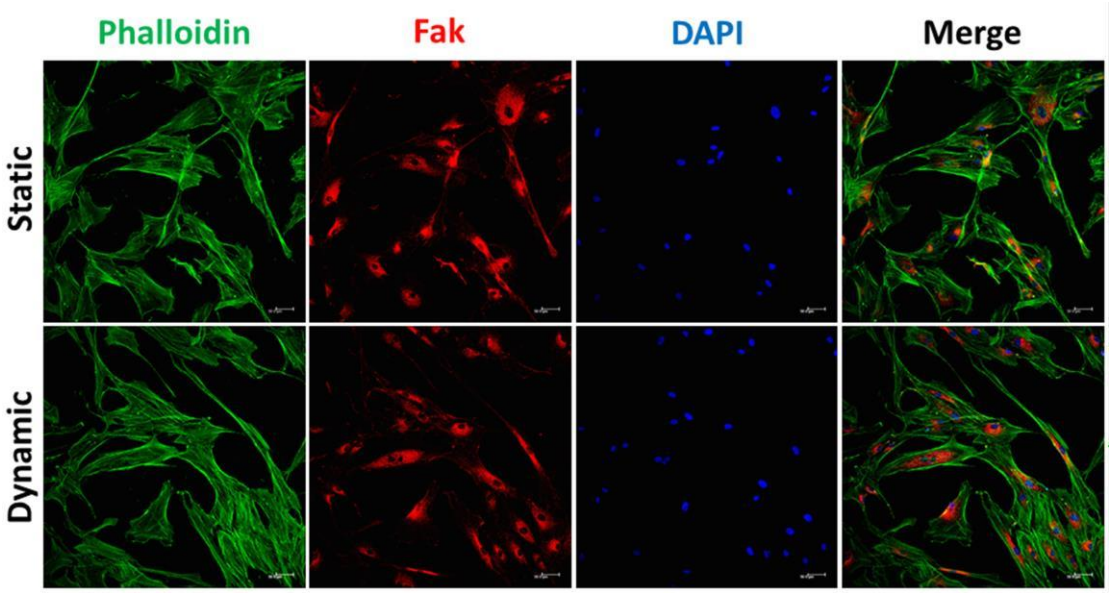
SUPLEMENTARY FIGURES

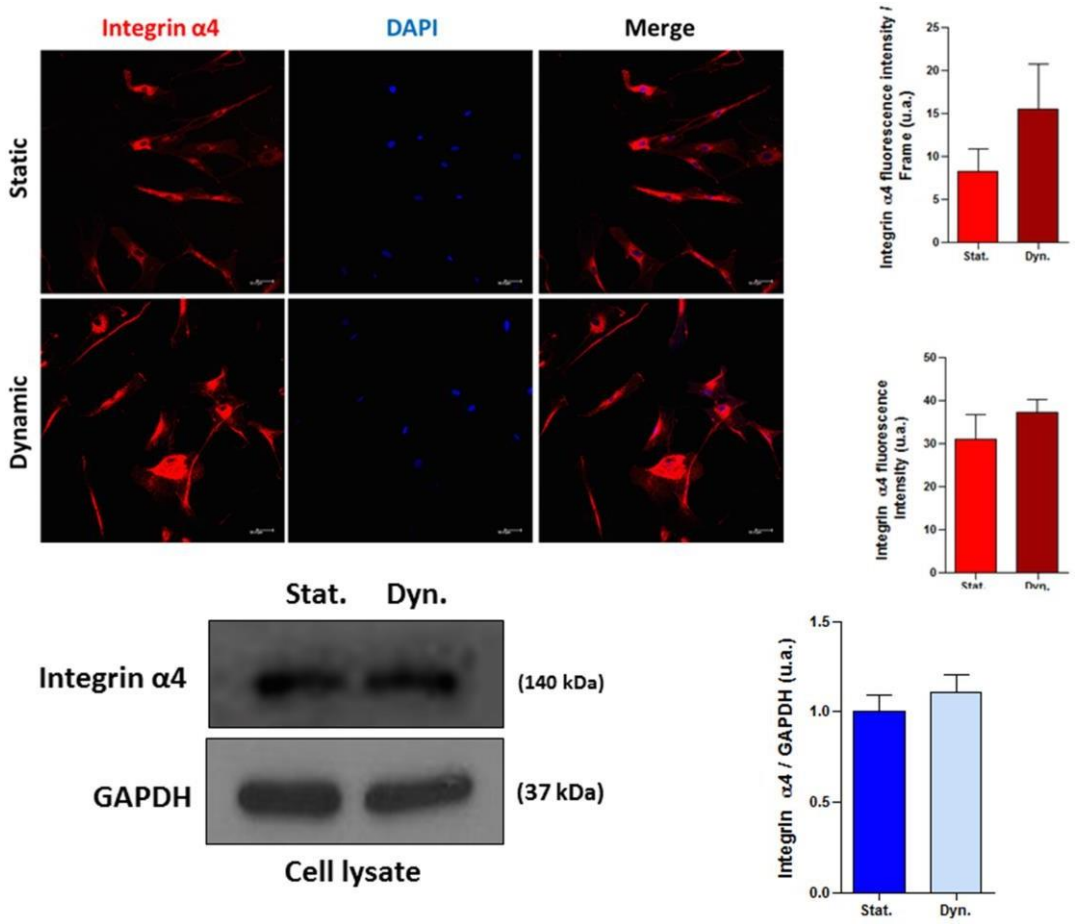
Laminar shear stress-provoked cytoskeletal changes are mediated by epigenetic reprogramming of *TIMP1* in human primary smooth muscle cells

Fig. S1.



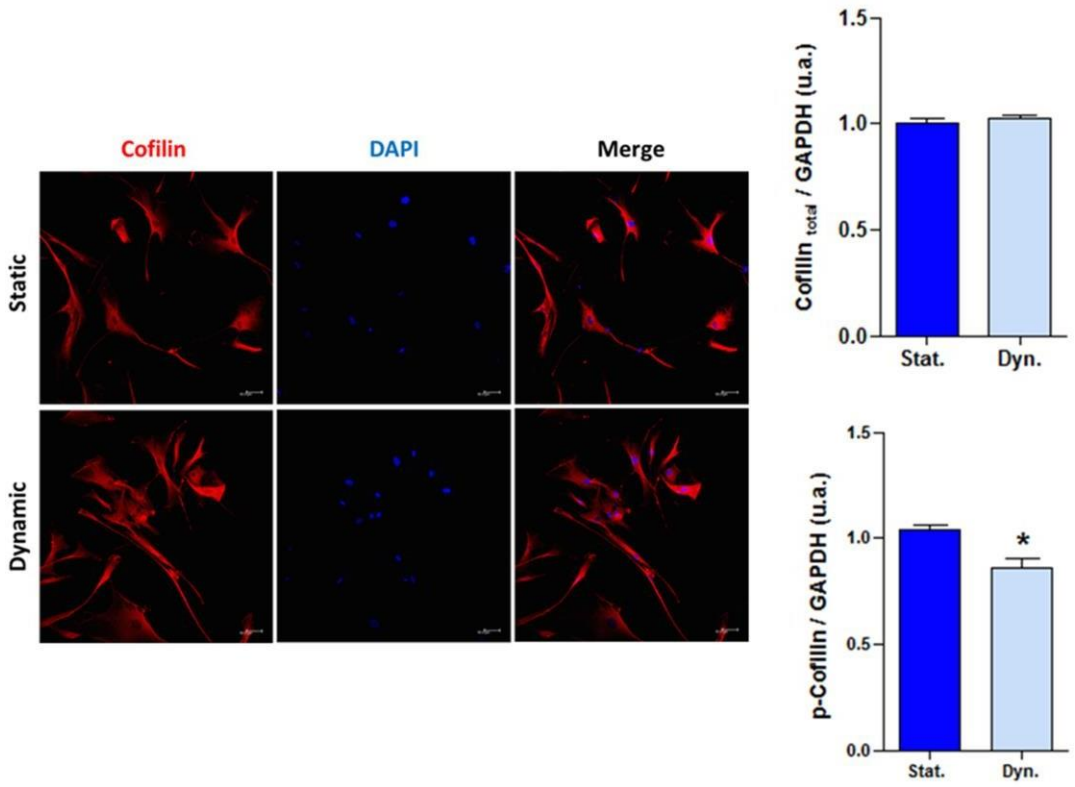
FAK subcellular distribution and the actin filaments organization (F-actin) were evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and Alexa Fluor 488-conjugated phalloidin (green). The nuclei were stained with DAPI (blue). Images are representative of three independent experiments. Bar = 50 µm.

Fig. S2.



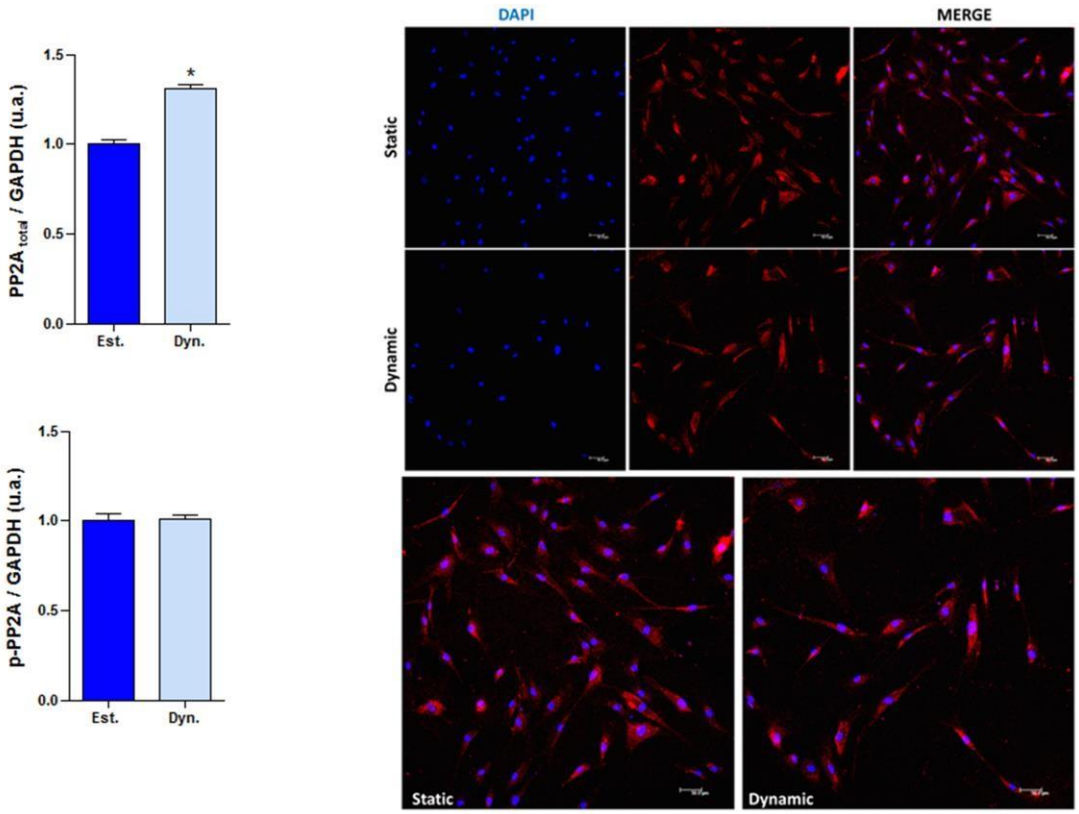
Integrin α4 subcellular distribution was evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and nuclei were stained with DAPI (blue). Intensity fluorescence was analysed using the program LAS AF and was presented one representative immunoblot of Integrin α4. Densitometric analysis of immunoblots was analysed using the program ScionImage and normalized to the protein ratio of controls (1), GAPDH was used as loading control. Images are representative of three independent experiments. Bar = 50 μm. Results were represented as mean ± standard deviation of three independent experiments.

Fig. S3.



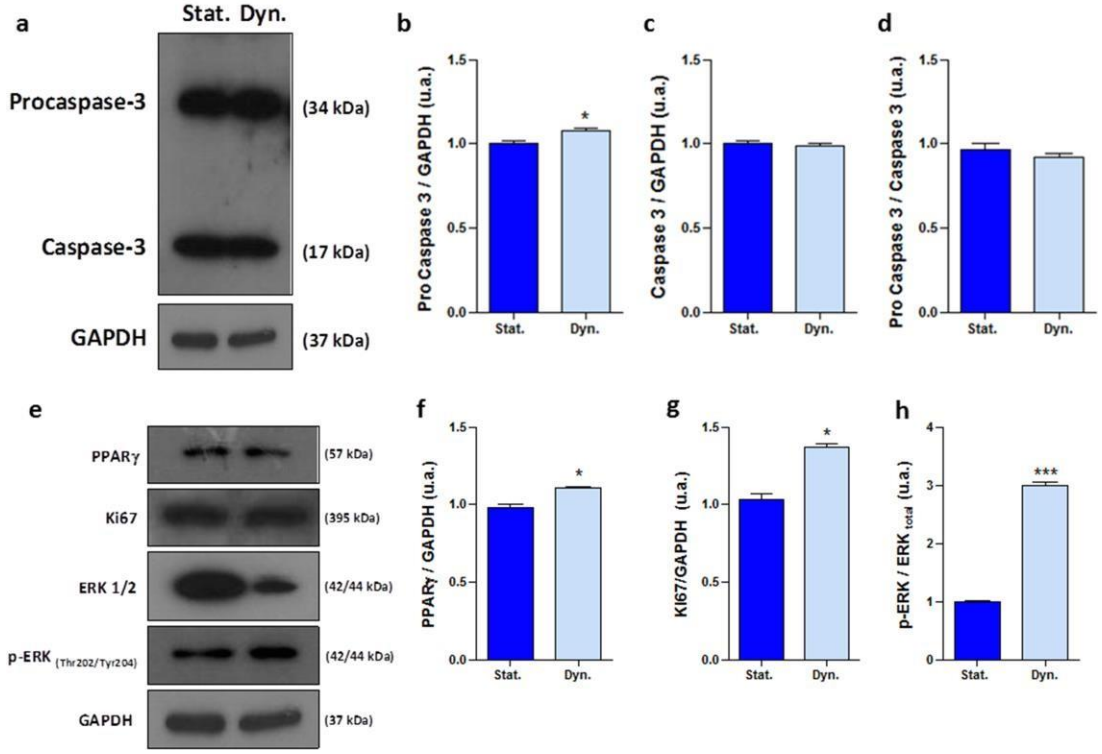
Cofilin subcellular distribution and the actin filaments organization (F-actin) were evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and Alexa Fluor 488-conjugated phalloidin (green). The nuclei were stained with DAPI (blue). Intensity fluorescence was analysed using the program LAS AF and the images are representative of three independent experiments. Bar = 50 µm. Results were represented as mean ± standard deviation of three independent experiments. *P < 0.05 compared with Static.

Fig. S4.



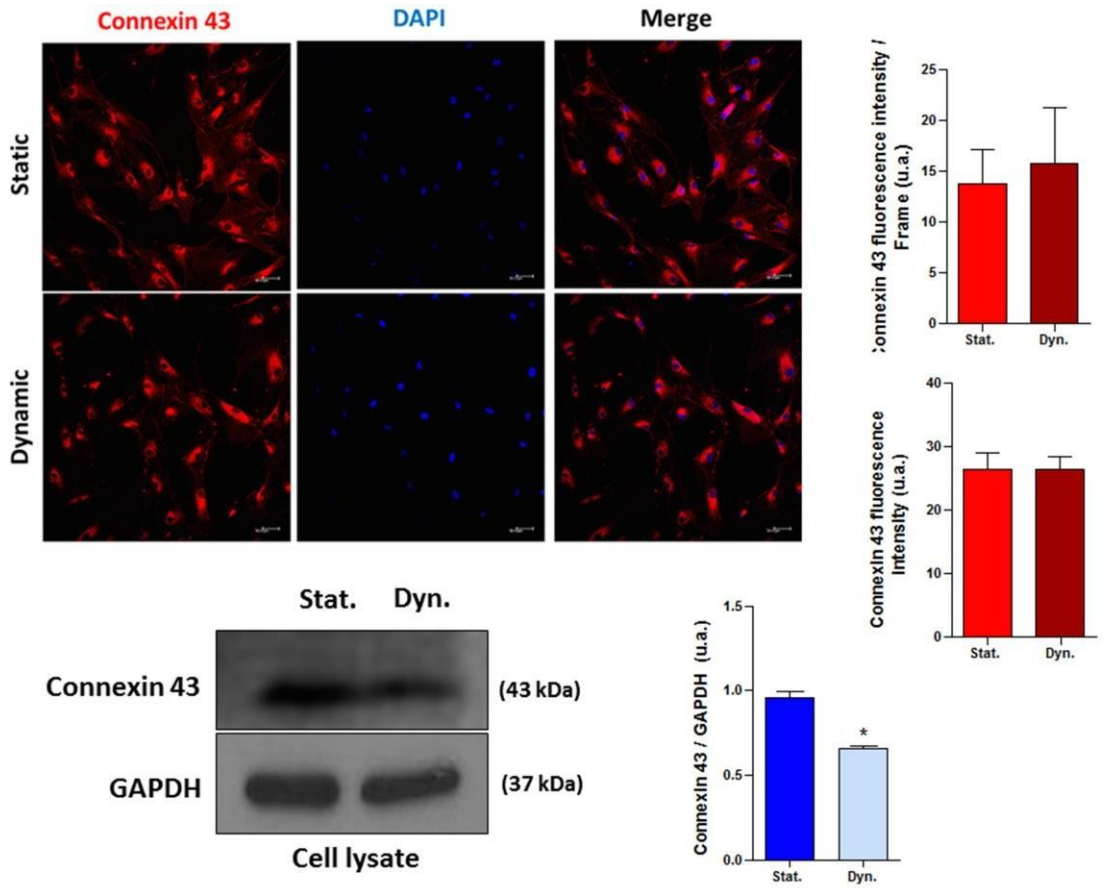
β-Catenin subcellular distribution and the actin filaments organization (F-actin) were evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and Alexa Fluor 488-conjugated phalloidin (green). The nuclei were stained with DAPI (blue). Images are representative of three independent experiments. Bar = 50 µm. Densitometric analysis of total and phosphorylated PP2A immunoblots was analysed using the program ScionImage and normalized to the protein ratio of controls (1), GAPDH was used as loading control. Results were represented as mean ± standard deviation of three independent experiments. *P < 0.05 compared with Static.

Fig. S5.



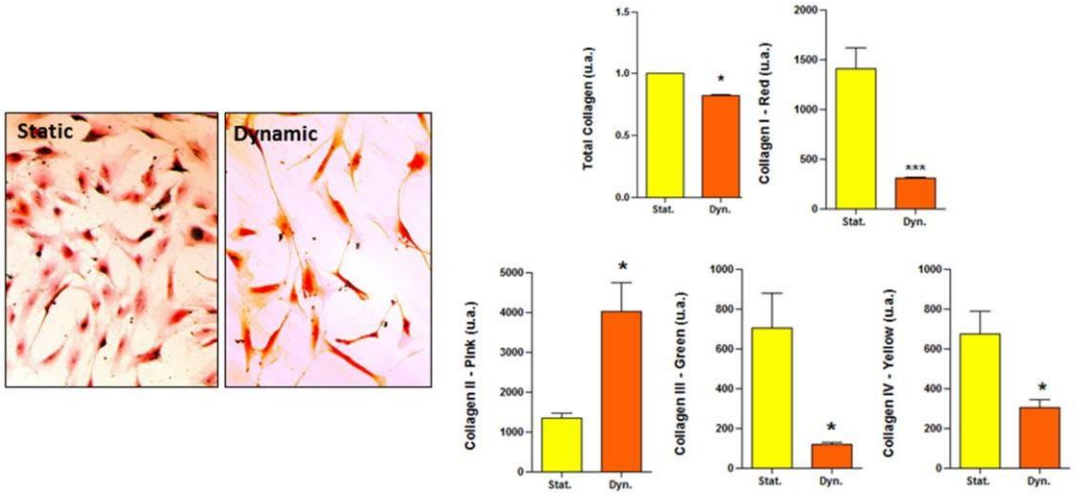
For the analysis of the levels or phosphorylation status of endogenous protein, equal amounts (75 µg) were loaded per lane and blotted with specific antibodies. One representative immunoblot of total cell lysates (a and e) is presented. The analyse was performed using the program ScionImage and normalized to the protein ratio of controls (1), GAPDH was used as loading control (b, c, d, f, g and h). Results were represented as mean ± standard deviation of three independent experiments. *P < 0.05 and ***P < 0.0001 compared with Static.

Fig. S6.



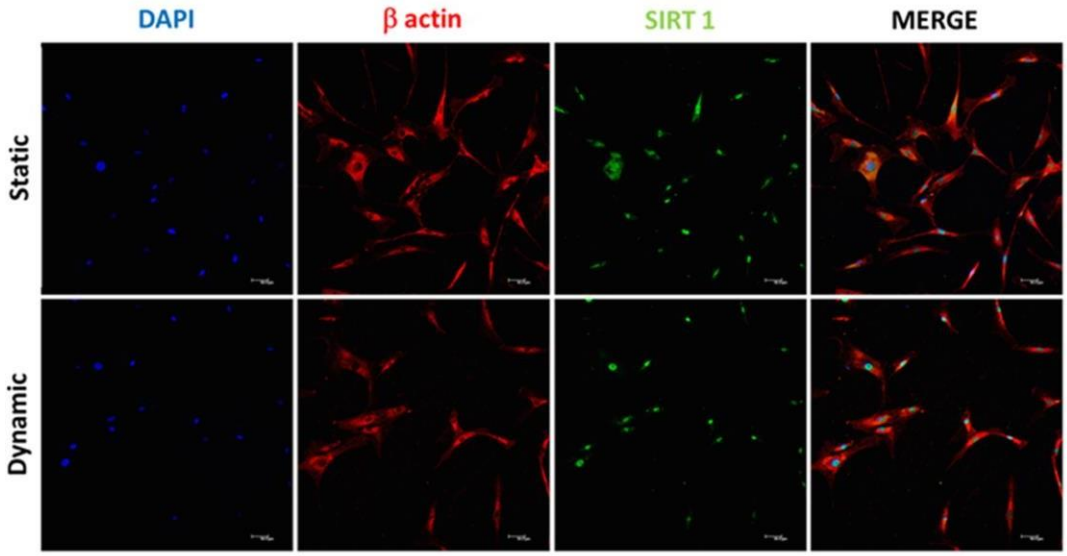
Connexin 43 subcellular distribution was evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) and nuclei were stained with DAPI (blue). Intensity fluorescence was analysed using the program LAS AF and was presented one representative immunoblot of Connexin 43. Densitometric analysis of immunoblots was analysed using the program ScionImage and normalized to the protein ratio of controls (1), GAPDH was used as loading control. Images are representative of three independent experiments. Bar = 50 µm. Results were represented as mean ± standard deviation of three independent experiments. *P < 0.05 compared with Static.

Fig. S7.



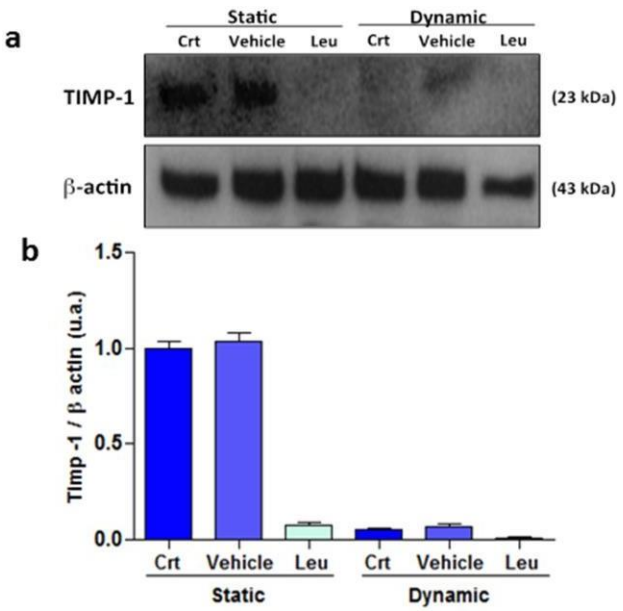
For quantitative analysis of collagen types the mechanically stressed AoSMC was stained with picrosirius red and the number of spots in the area recorded in the photo was determined using the ImageJ Software, adjusting the Threshold to identify the total collagen and our subtypes. For confocal microscopy analysis, the cells were examined using an inverted laser scanning confocal microscope Leica TCS SP5 (LEICA, Germany). Images are representative of three independent experiments and results were represented as mean ± standard deviation of three independent experiments. *P < 0.05 compared with Static. Bar = 50 µm.

Fig. S8.



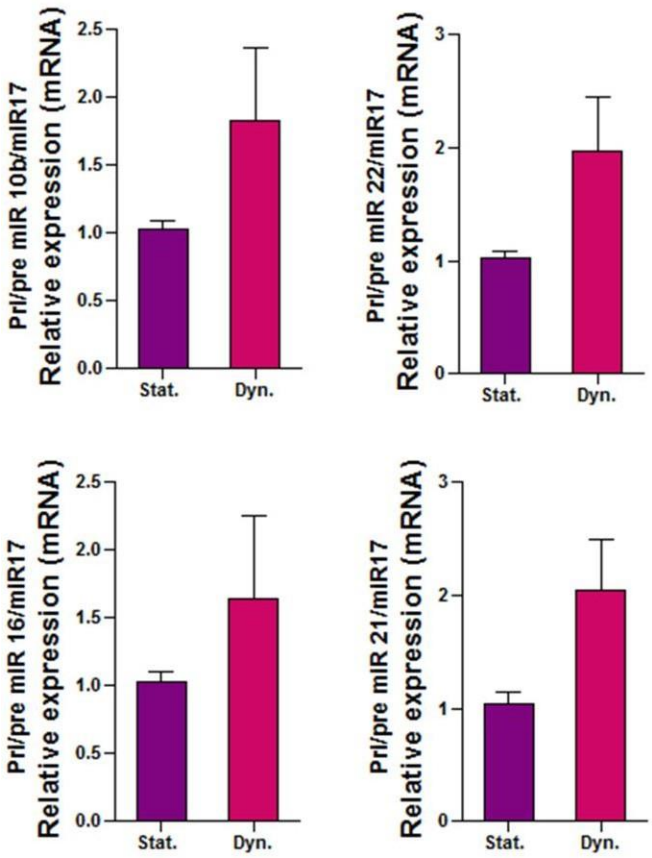
SIRT1 SUBCellular distribution was evaluated by laser confocal microscopy after incubation of the mechanically stressed AoSMC with specific antibody, followed by staining with Alexa Fluor 488 goat anti-rabbit IgG antibody (green) and t*he membrane was* staining with antibody for β-actin, followed by Alexa Fluor 594 goat anti-mouse IgG antibody (red). The nuclei were stained with DAPI (blue). Images are representative of three independent experiments. Bar = 50 μm.

Fig. S9.



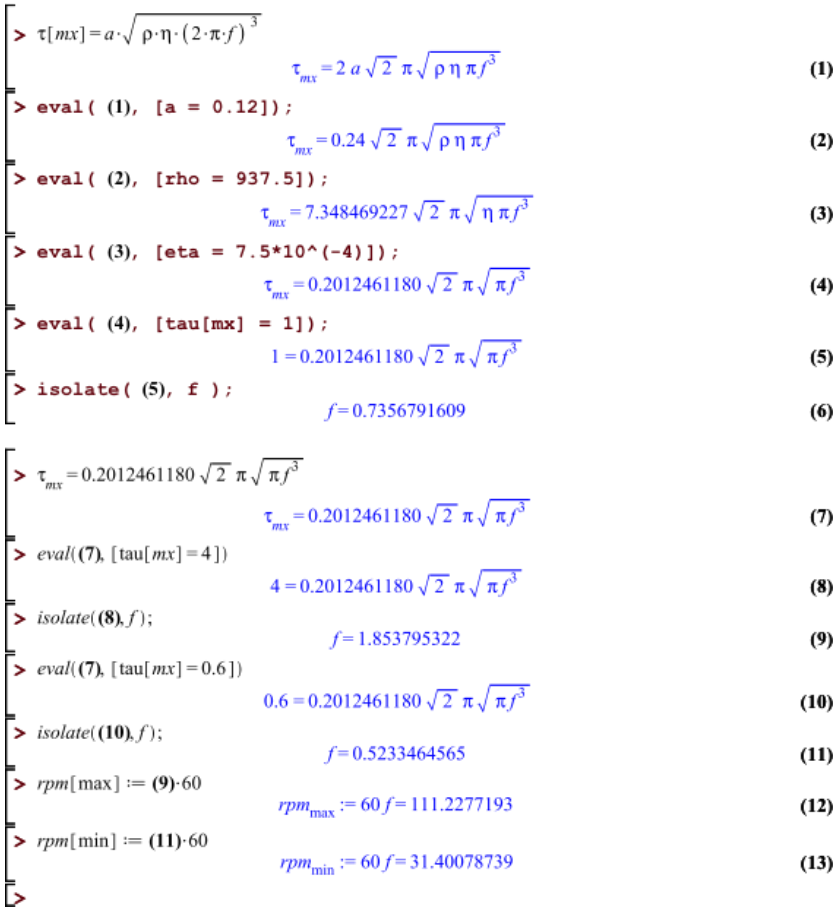
Proteasome-independent TIMP1 protein down-regulation. Levels endogenous of TIMP-1 were determined in AoSMC tretated-proteasome (Leu) inhibitor by immunoblotting (a). For p*roteasome inhibition was added 5* μM of Z-Leu-Leu-Leu-al (SIGMA, C2211) in the medium and the cells were submitted properly to shear stress protocols up to 24 hours at a rotation frequency of 100 rpm, as detailed previously. Densitometric immunoblotting analysis expressed as the relative intensity of TIMP1 (b), normalized by β-actin (housekeeping). Briefly, the proteasome inhibitor did not promote any TIMP1 recovering, suggesting this biological event being a proteasome-independent pathway.

Fig. S10.



Pri/pre miRNAs expression was detected by real time PCR analysis normalized by miR17 expression level. Results were represented as mean ± standard deviation of three independent experiments.

Fig. S11.



Equation (**1**) was obtained in the article de la Paz et al. (2012), relating the physiological shear stress (τmx) with the stirring plate radius (**a**), medium density (ρ), viscosity of the medium (η) and the frequency of rotation applied (**f**). The radius of the plate is 0.12 m, the density of the medium is 937.5 kg/m3 and the viscosity is 75,000 Ns/M2 (Equations (**2**), (**3**) and (**4**)). The physiological shear stress was set as τmx= 4 N/M2 for max value and τmx= 0.6 N/M2 for min value (Equations (**8**) and (10)). Then the frequency for plate rotation was obtained as f = 111.28 rpm (**12**) and f = 31.40 (**13**). These values define the range of rpm applicable to the plate as 31.40 – 111.28.

CAPÍTULO 4

DISCUSSÃO GERAL, CONSTAÇÕES & CONCLUSÃO

DISCUSSÃO GERAL

Cada vez mais tem-se buscado compreender mecanismos celulares envolvidos com respostas ao microambiente, a fim de alicerçar mecanismos comparativos em situação de desordem tecidual/fisiológica, onde capacita pesquisas translacionais a buscar determinados biomarcadores e alvos terapêuticos. Neste sentido, muito se discutido acerca de mecanismos epigenéticos como respostas imediatas ao ambiente e capaz de modular a expressão gênica, interferindo, assim, no fenótipo celular. Nesta dissertação, buscamos compreender eventos celulares/bioquímicos envolvidos com a capacidade adaptativa de células endoteliais e de musculatura lisa à tensão de cisalhamentos (*Shear-Stress*). Para isso, um modelo proposto na literatura foi modificado afim de mimetizar estas forças tensionais. Aqui, uma reflexão é importante: métodos alternativos *in vitro* que mimetizem eventos biológicos satisfaz uma necessidade nobre de reduzir o número de animais de experimentação. Assim, destacamos o modelo utilizado neste estudo, onde células aderidas ao fundo de uma placa de petri modificada foram submetidas à forças laminares tensionais, onde a intensidade do fluxo pode ser criticamente variada; haja vista nossa recente publicação onde mostramos variações tensionais em um circuito experimental de *shear-stress*, mimetizando um ambiente hipertensivo. Células submetidas nesse modelo experimental foram devidamente coletadas e tratadas para os diferentes protocolos e abordagens metodológicas.

De um modo geral, nossos resultados mostram características comuns entre células endoteliais e de musculatura lisa, como a necessidade dinâmica de mecanismos epigenéticos capazes de reger eventos adaptativos onde um balanço de metilação global pode oferecer pistas importantes do estado transcricional destas células. Especialmente quanto às células endoteliais, mostramos haver eventos que favorecem mecanismos epigenéticos de manutenção da metilação, diminuindo o perfil de hidroxilação. Notamos que estes eventos foram concomitantes ao remodelamento da matriz extracelular e, como a estabilidade da matriz dispara mecanismos intracelulares de sobrevivência e proliferação celulares, sugerimos estes eventos como iniciadores das respostas epigenéticas, onde a atividade de MMPs geram certa instabilidade do substrato e um novo processo adaptativo se orienta. Neste contexto, ainda, tomando como base dados de expressão gênica, sugerimos vias acessórias de adaptação envolvendo VEGFR e sinalização intracelular que leva ao aumento de NOS_2 e NOS_3; aqui, reportamos que esta via pode ter sido potencializada pela atividade de AKT.

Em relação às células musculares, destacamos a importância do remodelamento da matriz como um evento chave na adaptação celular ao *shear-stress* quando mostramos que o gene que codifica uma proteína envolvida no controle negativo da atividade de MMPs, a proteína TIMP, sofre regulação epigenética, através de miRs. Mostramos através de

diferentes metodologias a necessidade de se rearranjar o citoesqueleto a medida que a matriz extracelular era remodelada. Destacamos ainda que a quantidade de TIMP1 no meio extracelular de células desafiadas pelo *shear-stress* era significantemente inferior ao controle, onde as células eram mantidas em condições estáticas.

Por fim, destacamos que a metodologia proposta nos 2 trabalhos apresentados mimetiza o fluxo biológico do sangue e nos fornece condições importantes de controle experimental para buscar biomarcadores adaptativos deste processo, os quais poderão ser confortados e futuramente validados com modelos in vivo de distúrbios do fluxo, servindo-nos como alvos moleculares na busca por terapias mais eficientes.

CONSTATAÇÕES

1. Tecnicamente, o cultivo de células primárias é bastante dificultado quando se necessita experimentalmente de uma densidade celular elevada, visto seu baixo crescimento in vitro; mesmo seguindo toda recomendação do fabricante;

2. O shear stress provoca mudanças na maquinaria molecular que governa os fenótipos de sobrevivência e proliferação, intensificando o rearranjo do citoesqueleto;

3. O comportamento adaptativo das células endoteliais ao shear stress requer remodelação da MEC;

4. A mecanotransdução promove um panorama epigenética específico como potencial controle do fenótipo proliferativo e remodelamento da MEC;

5. Shear stress in vitro provoca adaptação fisiológica nas AoSMC;

6. A adaptação fisiológica em resposta ao shear stress resulta da expressão alterada de MMPs/TIMP1 e subsequente remodelamento da MEC;

7. A reprogramação epigenética medeia as respostas do shear stress em AoSMC;

8. A regulação negativa da proteína TIMP1 por células musculares lisas envolve alterações no controle epigenético desse gene.

CONCLUSÃO

Levando em conta nosso modelo experimental e limitações técnicas, concluímos que respostas adaptativas do endotélio, aqui avaliado *in vitro*, ao shear-stress requer um remodelamento dinâmico da matriz extracelular, concomitante ao rearranjo do citoesqueleto e ativação de vias de sobrevivência e proliferação celulares, o que sugere modulação epigenética dinâmica.

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