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Efeito do resveratrol sobre parâmetros de ativação de células GRX

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Efeito do resveratrol sobre parâmetros de ativação de células GRX

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Bioquímica.

Orientador (a): Prof. Dra. Fátima T.C.R. Guma

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2020

“Aquele que tem uma razão para viver pode suportar quase tudo. ”

Friedrich Nietzsche

“O mais competente não discute, domina a sua ciência e cala-se.”

Voltaire

“Muita coisa que ontem parecia importante ou significativa amanhã virará pó no filtro da memória. Mas o sorriso (...) ah, esse resistirá a todas as ciladas do tempo. ”

Caio Fernando Abreu

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À minha família pelo apoio durante este árduo caminho. Muito obrigado!!!

À **CAPES**, pela bolsa de doutorado.

Apresentação

Esta tese está organizada em seções dispostas em: Parte I (Resumo, Abstract, Introdução e Objetivos), Parte II (Capítulos [I, II e III referentes aos artigos científicos]) e Parte III (Discussão, Conclusão, Perspectivas e Referências).

A Parte I incluem a Introdução, onde apresentamos o embasamento teórico destes necessários para esta tese, bem como seus objetivos.

A Parte II contém os artigos científicos deste doutorado, sendo um já publicado e dois em preparo para submissão. Em cada artigo constam os objetivos, materiais e métodos e referências utilizadas. Os experimentos foram realizados nos laboratórios dos professores: Profa. Dra. Fátima Costa Rodrigues Guma (Lab. 21 do PPG Bioquímica/UFRGS) e Jarbas Rodrigues de Oliveira (Laboratório de Pesquisa em Biofísica Celular e Inflamação da PUCRS).

A Parte III inclui a Discussão, Conclusões e Perspectivas, que contêm uma interpretação geral dos resultados obtidos nos diferentes artigos que formam esta tese, as conclusões gerais obtidas nesta tese e as perspectivas futuras possibilidades de desenvolvimento de projetos a partir dos resultados obtidos para continuidade desta linha de pesquisa.

O tópico Referências reúne a lista resultante do referencial teórico utilizado na confecção das seções Introdução e Discussão desta tese.

Sumário

Lista de abreviaturas e siglas

MEC - Matriz extracelular

HSC - Célula estrelada hepáticas

HCV - Vírus da hepatite C

NASH - Esteato-hepatite não alcoólica

GRX – Granuloma x

LSECs - Células endoteliais sinusoidais do fígado

TIMPs - Inibidores teciduais de metaloproteinases

MMPs - Metaloproteinases da matriz

aHSC – Célula estrelada hepática ativada

qHSC – Célula estrelada hepática quiescente

GEO - Gene Expresion Omnibus

David - Database for Annotation, Visualization, and Integrated Discovery

KEGG - Kyoto Encyclopedia of Genes and Genomes

TGFB1 - Transforming growth factor beta-1

SIRT1 – Sirtuina 1

PPAR - proliferadores de peroxissoma

PPARγ - proliferadores de peroxissoma tipo gama

PPARα - proliferadores de peroxissoma tipo alfa

CCl_4 – Tetracloreto de carbono

αSMA - alfa actina de músculo liso

COL1A1 - Colágeno tipo I alfa 1

TNFα – Fator de necrose tumoral alfa

IL-6 - Interleucina 6

FFA - Àcidos graxos livres

RSV – Resveratrol

TGF-β1 **-** Fator de transformação do crescimento beta1

NF-κB – Fator nuclear kappa B

IL-10 – Interleucina 10

LPS – Lipopolissacarídeo

FDA - Administração de Alimentos e Medicamentos

EMA - Agência Europeia de Medicamentos

HGF - Fator de crescimento de hepatócitos

GFAP - Proteína glial fibrilar ácida

DEG - Gene de expressão diferencial

PPI - Interação proteína-proteína

GO - Ontologia genética

LSEC Células endoteliais sinusoidais do fígado

LISTA DE FIGURAS

RESUMO

A fibrose e/ou a cirrose hepática são doenças crônicas do fígado e representam uma das maiores causas de mortalidade humana. As células estreladas hepáticas (HSC) são protagonistas desse processo e estão associadas ao desenvolvimento da fibrose que, em último estágio, acarreta em cirrose. No fígado saudável, estas células apresentam um fenótipo quiescente ou lipocítico, caracterizado pela sua capacidade de armazenar gotas lipídicas. Entretanto, danos contínuos ao fígado desencadeiam uma resposta que gera estímulos autócrinos e parácrinos mediados por citocinas e espécies reativas de oxigênio. Este quadro induz uma modulação das HSC ao fenótipo ativado ou miofibroblastóide, caracterizado pelo aumento da capacidade de produzir componentes de matriz extracelular, cuja deposição exagerada configura o estado patológico da fibrose. A fitoalexina Resveratrol (3,5,4'-tetrahidroxistilbeno; RSV) tem sido relacionada a inúmeros efeitos benéficos à saúde por suas atividades de citoproteção e quimioprevenção. Há evidencias que suas propriedades que podem ser benéficas no tratamento da fibrose hepática. Nesta tese, avaliamos os efeitos do RSV em alguns marcadores de ativação em células da linhagem GRX. Na sequência, exploramos, por meio de recursos integrados de bioinformática e da utilização de bancos de dados públicos, as possíveis proteínas alvo do RSV e seus papéis potenciais na modulação fenotípica de HSC. Por fim, com ferramentas de bioinformática buscamos identificar variações na expressão genica e rotas metabólicas que atuam na transdiferenciação das HSC. Nossos resultados demonstram que o tratamento com 50 µM de RSV por 24 horas aumentou o conteúdo dessas proteínas relacionadas à ativação. Além disso, o RSV não alterou a morfologia semelhante a miofibroblastos de GRX. Curiosamente, o RSV a 10 e 50 µM diminuiu a migração de GRX e a contração do gel de colágeno I. Mostramos que o RSV desencadeou o aumento do conteúdo de TNF-α e IL-10 nos meios de cultura de GRX, enquanto o contrário ocorreu para o conteúdo de IL-6. A maioria dos genes alvos do RSV estão associados a várias vias celulares como: Via de transcrição genérica, Transcrição de RNA polimerase II, Interleucina-4 e Interleucina-13. Ainda, foram identificados 26 DTPs (Direct Target Proteins) no banco de dados DRUGBANK. Em seguida, a rede de interação proteína-proteína (PPI) e as vias Reactome foram analisadas. Da mesma forma, foram buscados artigos científicos sobre os genes-alvo do RSV na base de dados PUBMED. Foram encontrados 26 DTPs de RSV. Descobrimos que apenas 7 DTPs já foram associados a estudos no banco de dados PUBMED. Bem como, na análise de expressão diferencial foram encontrados 411 genes sendo 155 superexpressos e 256 subexpressos. Em resumo, nossos resultados sugerem que o RSV não diminuiu o estado de ativação da GRX; ao contrário, desencadeou um efeito de pró-ativação. Na sequência por meio de recursos integrados de bioinformática e da utilização de bancos de dados públicos, analisamos as possíveis proteínas alvo do RSV e seus papéis potenciais na modulação fenotípica de HSC. Também, os principais genes regulados positivamente foram descritos na literatura como estando envolvidos na ativação de HSC ou no desenvolvimento de fibrose hepática.

Palavras chaves: Bioinformática; células estreladas hepáticas, fibrose hepática, resveratrol

ABSTRACT

Fibrosis and / or liver cirrhosis are chronic liver diseases and represent the major cause of human mortality. Hepatic stellate cells (HSC) are protagonists in this process and are associated to the development of fibrosis, which, at the last stage, causes cirrhosis. In healthy liver, these cells present a quiescent or lipocytic phenotype, characterized by their ability to store lipid droplets. However, continuous damage to the liver triggers a response that generates autocrine and paracrine stimuli mediated by cytokines and reactive oxygen species. This condition induces the HSC modulation to the activated or myofibroblastoid phenotype, characterized by the increased capacity to produce components of extracellular matrix, whose excessive deposition configures the pathological state of fibrosis. The phytoalexin Resveratrol (3,5,4'-tetrahydroxystilbene; RSV) has been linked to numerous beneficial health effects due to its cytoprotection and chemoprevention activities. There is evidence that RSV properties may be beneficial in the treatment of liver fibrosis. In this thesis, we evaluated the effects of RSV on some activation markers of the GRX cell line. Further, we explored, using integrated bioinformatics resources, the possible RSV target proteins and their potential roles in the phenotypic modulation of HSC. Finally, with bioinformatics tools, we seek to identify variations in gene expression and metabolic routes that act in the transdifferentiation of HSC. Our results demonstrated that treatment with 50 µM of RSV for 24 hours increased the content of these proteins related to activation. In addition, RSV did not alter the GRX myofibroblast-like morphology. Interestingly, RSV at 10 and 50 µM decreased GRX migration and contraction of collagen I gel. We showed that RSV triggered an increase in the content of TNF-α and IL-10 in GRX culture media, while the opposite occurred for IL-6 content. Most of the target genes for RSV are associated with several cellular pathways such as: Generic transcription pathway, RNA polymerase II transcription, Interleukin-4 and Interleukin-13. In addition, 26 DTPs (Direct Target Proteins) were identified in the DRUGBANK database. Then, the protein-protein interaction network (PPI) and the Reactome pathways were analyzed. Likewise, scientific articles on RSV target genes were searched for in the PUBMED database. 26 RSV DTPs were found. We found that only 7 DTPs have been associated with studies in the PUBMED database. As well as the analysis of differential expression, 411 genes were found, 155 overexpressed and 256 underexpressed. In summary, our results suggest that RSV did not decrease the GRX activation state; on the contrary, it triggered a pro-activation effect. Following through integrated bioinformatics resources, the possible RSV target proteins and their potential roles in the phenotypic modulation of HSC. Also, the main positively regulated genes have been described mainly in the literature as being involved in the activation of HSC or in the development of liver fibrosis.

Keywords: Bioinformatics, hepatic stellate cells, liver fibrosis, resveratrol

Parte I

1 INTRODUÇÃO

1.1 Fibrose Hepática

Fibrose hepática é definida como o resultado de um desequilíbrio entre a síntese e a degradação da matriz extracelular (MEC) produzida por fibroblastos do tecido conjuntivo hepático e pela célula estrelada hepáticas (do inglês – *Hepatic stellate cells,* HSC) ativadas (Reeves and Friedman 2002). Acredita-se que a fibrose, que foi definida em 1978 pela Organização Mundial da Saúde, é uma característica comum a muitas doenças crônicas do fígado (Friedman 2007). As doenças crônicas do fígado representam uma grande preocupação para a saúde pública em todo o mundo, com mais de 100 milhões pessoas afetadas e uma taxa de mortalidade de aproximadamente 2 milhões de mortes por ano (Li, Fan et al. 2011, Byass 2014, Marcellin and Kutala 2018). Em especial, a cirrose é o estágio terminal da fibrose hepática, representa 1% a 2% da população global é acometida por esta condição, resultando em mais de 1 milhão de mortes anualmente em todo o mundo (Collaborators, Forouzanfar et al. 2015, Collaborators 2016).

O fígado é um órgão dinâmico que exerce um importante papel na regulação do metabolismo e detoxificação do organismo; e este processo pode acarretar lesões no parênquima hepático. No caso de lesão hepática aguda, o fígado tem um potencial para restabelecer rapidamente a integridade estrutural hepática. No entanto, estímulos nocivos sustentados cronicamente levam à alteração do parênquima hepático com progressiva deterioração de sua função (Friedman 2007). O processo de fibrogênese hepática constitui uma resposta a uma persistente lesão celular devido a uma reação inflamatória crônica que induz uma ativação de células fibrogênicas

(HSC), morte de hepatócitos e o acúmulo de MEC (Figura 1) (Higashi, Friedman et al. 2017).

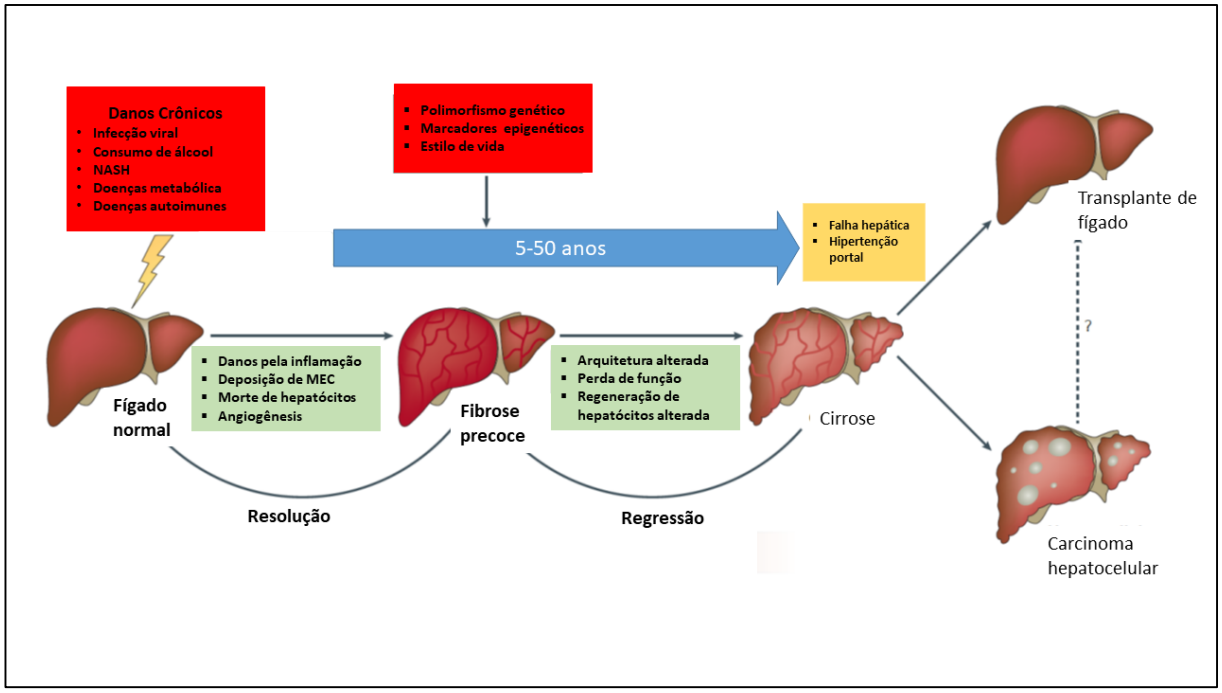

Figura 1: Processo de fibrose crônica hepática, adaptado de Pellicoro, 2014.

Em países industrializados, as principais causas da fibrose incluem: infecções pelo vírus da hepatite C (HCV), abuso no consumo de álcool e a esteato-hepatite não alcoólica (NASH, do inglês *Non-alchoolic steatohepatitis*). Outras causas subjacentes são insultos induzidos por toxinas (por exemplo: álcool ou drogas), distúrbios colestáticos, hepatite autoimune e doenças metabólicas hereditárias (Figura 2) (Hernandez-Gea and Friedman 2011).

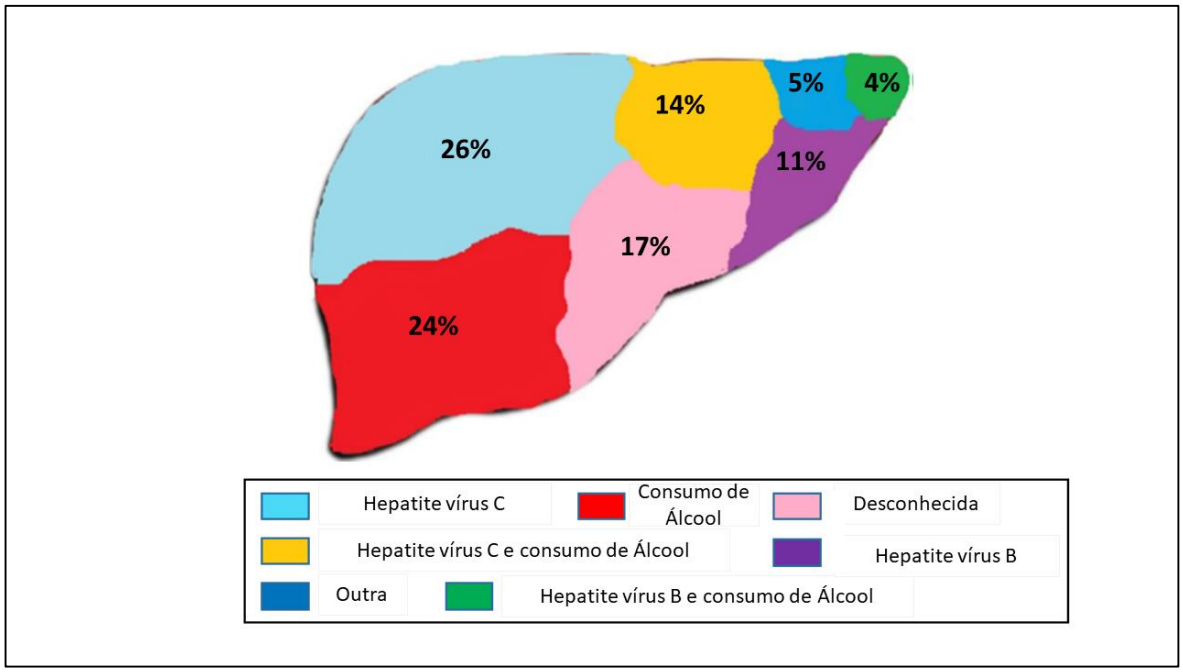

Figura 2: Causas de doenças crônicas do fígado, adaptado de Poilil Surendran et al (2017).

Tais condições podem culminar com o acúmulo de proteínas de matriz extracelular que, consequentemente, alteraram a arquitetura hepática devido à formação de cicatrizes fibrosas e subsequente desenvolvimento de nódulos de hepatócitos em regeneração, característicos da cirrose (Friedman 2007, Friedman 2008). Estas alterações podem ser fatais, pois podem resultar em complicações como hipertensão portal com sangramento por varizes, insuficiência hepática aguda-crônica e aumento do risco de carcinoma hepatocelular (Zoubek, Trautwein et al. 2017). As causas da cirrose hepática são multifatoriais e existem algumas características patológicas comuns a todos os casos de cirrose hepática, incluindo: degeneração e necrose de hepatócitos, substituição do parênquima hepático por tecidos fibróticos e nódulos regenerativos e perda da função hepática (Zhou, Zhang et al. 2014).

O processo de fibrose hepática pode ser dividido em três estágios: uma fase pré-inflamatória com ativação das HSC mediada por hepatócitos danificados; uma fase inflamatória, onde as HSC são estimuladas a transdiferenciar em miofibroblastos;

e uma fase pós-inflamatória, quando as HSC secretam citocinas estimulantes e componentes da MEC. Essas citocinas podem estimular miofibroblastos e HSC, criando um *feedback* positivo que perpetua o processo fibrogênico. A partir deste quadro, a resolução da fibrose refere-se a vias que causam tanto apoptose, senescência ou retorno a quiescência das HSC (Parsons, Takashima et al. 2007, Friedman 2008).

O destino das HSC quando a fibrose e/ou a cirrose hepática se estabelecem ainda não é claro, e não há um tratamento padrão para estas doenças. No entanto, nos últimos 30 anos, houve crescimento no número de trabalhos científicos acerca da fibrose hepática. Estes trabalhos, têm buscado revelar alvos potenciais para o tratamento desta enfermidade. Tais alvos incluem a indução das células ativadas para o fenótipo quiescente por meio da neutralização das respostas proliferativas, fibrogênicas e contráctil das HSC, a promoção da degradação de matriz extracelular e o estímulo a apoptose, a modulação das citocinas envolvidas, também, a redução dos níveis de estresse oxidativo (Bell, Sagare et al. 2007, Higashi, Friedman et al. 2017).

1.2 Fibrose Hepática e inflamação

Lesões inflamatórias hepáticas crônicas resultam em acúmulo de matriz extracelular e fibrose hepática, eventualmente levando à cirrose (Higashi, Friedman et al. 2017). O dano dependendo do tipo de lesão hepática subjacente apresenta vários mecanismos para desencadear reações imunológicas. Em especial as reações imunológicas crônicas culminam em fibrose hepática. Desta forma, compreender o mecanismo de inflamação e fibrose é extremamente importante para o

desenvolvimento de tratamentos para doenças hepáticas crônicas (Tanaka and Miyajima 2016).

Como um dos fatores patogênicos, a inflamação desempenha um papel predominante na instalação da fibrose hepática via comunicação e interação entre células inflamatórias, citocinas e as vias de sinalização relacionadas (Chen, Brenner et al. 2019). Durante a infecção os hepatócitos danificados, em especial, induzem um aumento dos fatores pró-inflamatórios, assim desencadeando o desenvolvimento de um quadro inflamatório (Friedman 2008). Ademais, já foi relatado que as vias de sinalização relacionadas às respostas inflamatórias são as principais vias de transdução de sinal para o desenvolvimento de um quadro de fibrose hepática e ativação das HSC (Reeves and Friedman 2002, Puche, Saiman et al. 2013). Esta última possui redes reguladoras de *crosstalk* entre macrófagos residentes que levam à ativação das HSC e à produção de citocinas pró-inflamatórias, que por sua vez iniciam a resposta fibrótica (Bataller and Brenner 2005, Friedman 2007). Cabe salientar, que a ativação da via NF-κB em hepatócitos lesados induz a liberação de uma série de citocinas pró-inflamatórias e quimiocinas, como TNF-α, IL-6 e CCL_2, que medeiam a inflamação do fígado (Son, Iimuro et al. 2007). O TGF-β1 derivado do macrófago é o principal ativador das HSC, sendo o agonista fibrogênico conhecido mais potente (Hellerbrand, Stefanovic et al. 1999).

No processo de inflamação hepática, há uma intensa rede de comunicação celular, onde há citocinas pró-inflamatórias responsáveis pelos eventos característicos da fibrose. O TNFα é uma das citocinas protumorigênicas mais bem caracterizadas na hepatocarcinogênese, pois ativa as vias de sinalização NF-κB e JNK (Wang and Wang 2019). Esta citocina também tem um importante papel em

muitas formas de lesão hepática, onde atua de forma crucial no processo de ativação das HSC e na regeneração de hepatócitos (Sachdeva, Chawla et al. 2015, Wang and Wang 2019). O principal tipo celular para a liberação de TNF-α no fígado são as células de Kupffer, que liberam TNF-α quando ativadas por fatores liberados por outras células, como hepatócitos danificados e pela ocorrência de espécies reativas de oxigênio (ROS) (Sachdeva, Chawla et al. 2015). A redução da produção de TNF-α e/ou bloqueio de sua ação minimiza significativamente a lesão hepática causada pela toxicidade do álcool, overdose de paracetamol ou a lesão hepática associada a isquemia/reperfusão em modelos animais (Jaeschke, Gores et al. 2002, Diehl 2004).

Outra citocina importante no processo de fibrose hepática é a interleucina-6 (IL-6), que é uma potente citocina que exerce múltiplas funções no organismo (Tanaka, Narazaki et al. 2014). Em condições fisiológicas, a IL-6 é essencial para a homeostase do tecido hepático, a regeneração hepática, a defesa contra infecções e o ajuste fino das funções metabólicas (Schmidt-Arras and Rose-John 2016). A IL-6 é reconhecida como uma importante citocina pró-inflamatória, cuja expressão está associada a muitos distúrbios inflamatórios (Tanaka, Narazaki et al. 2014). Os níveis séricos de IL-6 aumentam rapidamente após a infecção ou inflamação, portanto, são usados na prática clínica como um marcador para detectar condições inflamatórias, especialmente sepse (Jekarl, Lee et al. 2013). Os níveis séricos e intra-hepáticos de IL-6 também estão fortemente elevados em pacientes com doenças hepáticas agudas e crônicas (Streetz, Tacke et al. 2003). Curiosamente, a IL-6 apresenta duas funções contrastantes: atua como uma citocina pró-inflamatória em modelos de doenças inflamatórias crônicas (Yakut, Ozkan et al. 2018) e, ao contrário, pode desencadear efeitos anti-inflamatórios nos casos de inflamação aguda (Bansal, Kovalovich et al. 2005, Iwahasi, Rui et al. 2020).

A IL-10 atua como um inibidor da autofagia. Esta interleucina interage com STAT3 para exercer uma função anti-fibrogênica diante da lesão hepática (Hu, Shi et al. 2016). Um estudo demonstrou que, na lesão hepática causada por galactosamina/lipopolissacarídeo (LPS), a administração de IL-10 recombinante diminuiu os níveis séricos de TNF-α e de alanina aminotransferase, além de diminuir a necrose de hepatócitos, a expressão de moléculas de adesão, a infiltração de neutrófilos e a letalidade (Louis, Le Moine et al. 1997). Além dos efeitos anti-inflamatórios, a IL-10 possui atividades regulatórias para a homeostase da MEC. *In vitro*, a IL-10 diminui a expressão do colágeno tipo I e aumenta a expressão da metaloprotease-1 e -3 da matriz em culturas de fibroblastos de pele (Reitamo, Remitz et al. 1994). *In vivo*, camundongos *knockout* para IL-10 foram capazes de desenvolver formação excessiva de cicatrizes na pele após exposição ao óleo irritante (Rennick, Davidson et al. 1995). Outro estudo demonstrou que na fibrose hepática induzida por tioacetamida, a terapia genética com IL-10 reverteu o processo fibrótico e preveniu a apoptose celular após a fibrose já ter sido estabelecida, sugerindo um potencial terapêutico para o tratamento com IL-10 (Hung, Lee et al. 2005).

1.3 Células Estreladas Hepáticas (HSC) e a Linhagem celular GRX

As HSC são as principais produtoras de colágeno no fígado, que possibilitou uma nova etapa de estudo sobre a patogênese, que abrangem o diagnóstico e as terapias para combater a fibrose hepática (Friedman 2007, Parsons, Takashima et al. 2007). As HSC são células mesenquimais que retêm características de fibroblastos residentes (inseridos na matriz estromal normal) e pericitos (ligados às células endoteliais dos capilares). Compreendem aproximadamente um terço das células não

parenquimatosas e 15% do total de células residentes no fígado humano normal (Friedman 2008).

As HSC residem em um espaço subendotelial, entre a superfície basolateral dos hepatócitos e o lado antiluminal da camada celular endotelial sinusoidal fenestrada (também conhecido como espaço de Disse) (Figura 3) (Hernandez-Gea and Friedman 2011, Heymann and Tacke 2016). Neste local, as HSC realizam trocas de biomoléculas entre o fluxo sanguíneo portal do trato gastrointestinal e os hepatócitos. Essa comunicação intercelular se dá por meio de mediadores solúveis e citocinas/quimiocinas com diversos tipos de células próximas, como hepatócitos, células epiteliais biliares, células progenitoras hepáticas (células ovais), células Kupffer (macrófagos residentes no fígado), macrófagos derivados da medula óssea, células endoteliais sinusoidais do fígado (LSEC, do inglês *Liver sinusoidal endothelial cells*), células imunes infiltradas e células nervosas (Friedman 2008, Heymann and Tacke 2016, Higashi, Friedman et al. 2017).

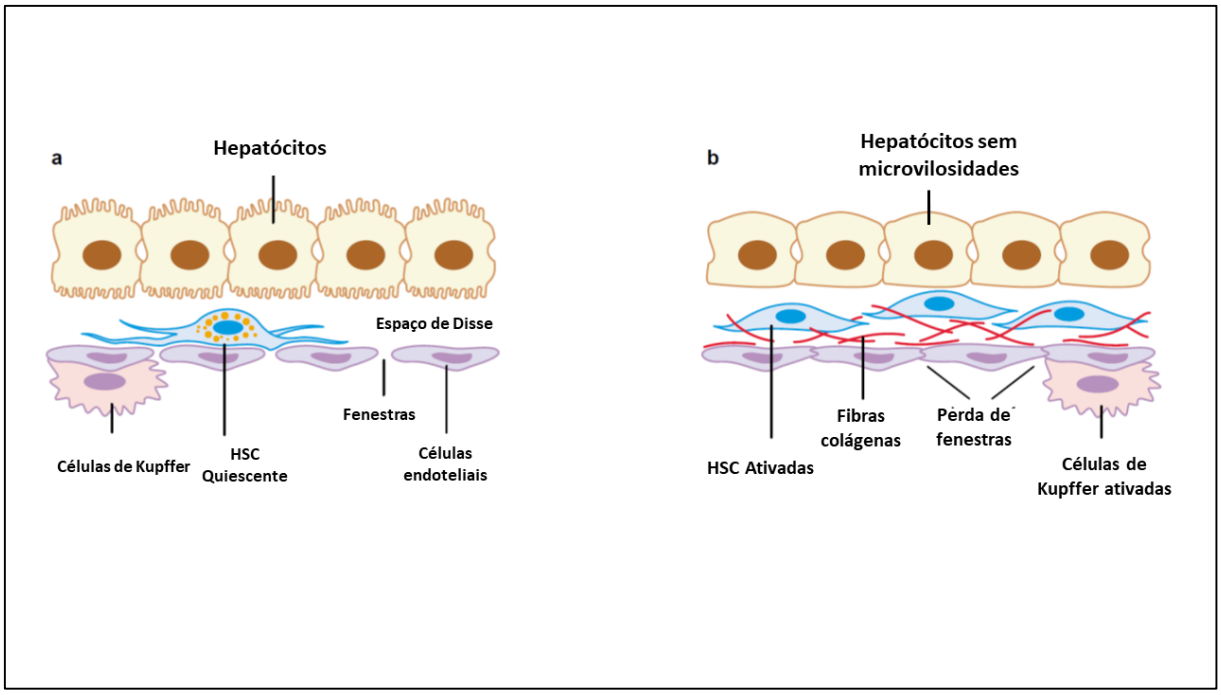

Figura 3: Localização das células estreladas hepáticas, adaptado de Huy, 2003. A) A localização das HSC quiescentes no espaço de Disse em um fígado normal. B) Mudanças na arquitetura hepática com o dano hepático e consequente ativação das HSC e deposição de MEC.

Em um fígado sadio, as HSC quiescentes, também chamadas de lipócitos, representam de 5 a 8% do total de células hepáticas e desempenham um importante papel no armazenamento e na liberação controlada de vitamina A, estocada como ésteres de retinol nas gotas lipídicas citoplasmáticas. As HSC também regulam a renovação da matriz extracelular pelo controle da secreção de metaloproteinases e de seus inibidores. Estas células são uma importante fonte de fatores parácrinos, autócrinos, justácrinos e quimioatratantes envolvidos na manutenção da homeostasia do espaço sinusoidal (Bataller and Brenner 2005) (Figura 4 A). A patogênese da fibrose é iniciada por um dano ao parênquima hepático seguido de um processo inflamatório e pró-oxidativo que leva a ativação das HSC (Bataller, Gasull et al. 2001, Geerts 2001). Neste contexto, as HSC quiescentes começam a proliferar e sofrer transdiferenciação em miofibroblastos contráteis em resposta à estimulação parácrina por tipos de células vizinhas, incluindo células Kupffer, hepatócitos, plaquetas,

leucócitos e células endoteliais sinusoidais (Bataller and Brenner 2005, Friedman 2008, Hernandez-Gea and Friedman 2011). Nesta condição, estas células passam a apresentar uma alteração no conteúdo e arranjo do citoesqueleto (estas células passam a apresentar filamentos de actina em feixes, caracterizando fibras de estresse), um aumento da taxa de proliferação, um desequilíbrio entre a síntese e degradação da matriz extracelular e, ainda, uma perda na capacidade de armazenamento de triacilgliceróis em gotas lipídicas, que serão utilizados como fonte de energia no processo de transdiferenciação (Hautekeete and Geerts 1997, Vogel, Piantedosi et al. 2000, Hernandez-Gea, Ghiassi-Nejad et al. 2012). As HSC ativadas são uma importante fonte de colágeno tipo I no fígado e podem secretar abundantemente proteínas da MEC. O desequilíbrio pró inibidores teciduais de metaloproteinases (TIMPs) e metaloproteinases da matriz (MMPs) provoca a remodelação da arquitetura hepática (Puche, Saiman et al. 2013, Higashi, Friedman et al. 2017). É importante ressaltar que as HSC são responsáveis por até 80% do total de colágeno fibrilar I no fígado fibrótico (Khomich, Ivanov et al. 2019), assim, a depleção das HSC ativadas é crítica para a resolução da fibrose (Figura 4B).

Neste contexto, vários modelos de cultura de células hepáticas *in vitro* estão sendo desenvolvidos para atender à necessidade de modelos preditivos no desenvolvimento e pesquisa de medicamentos, bem como, a análise e compreensão da complexidade da citobiologia hepática e de doenças associadas (Zeilinger, Freyer et al. 2016). Neste âmbito, a linhagem celular GRX é utilizada. Esta linhagem foi estabelecida a partir de granulomas produzidos em fígados de camundongos C3H/HeN, induzidos através de infecção experimental com *Schistossoma mansoni*. A cultura primária de células desses granulomas gerou esta linhagem de células

miofibroblastóides com características de células estreladas hepáticas no estado ativado (Borojevic, Monteiro et al. 1985).

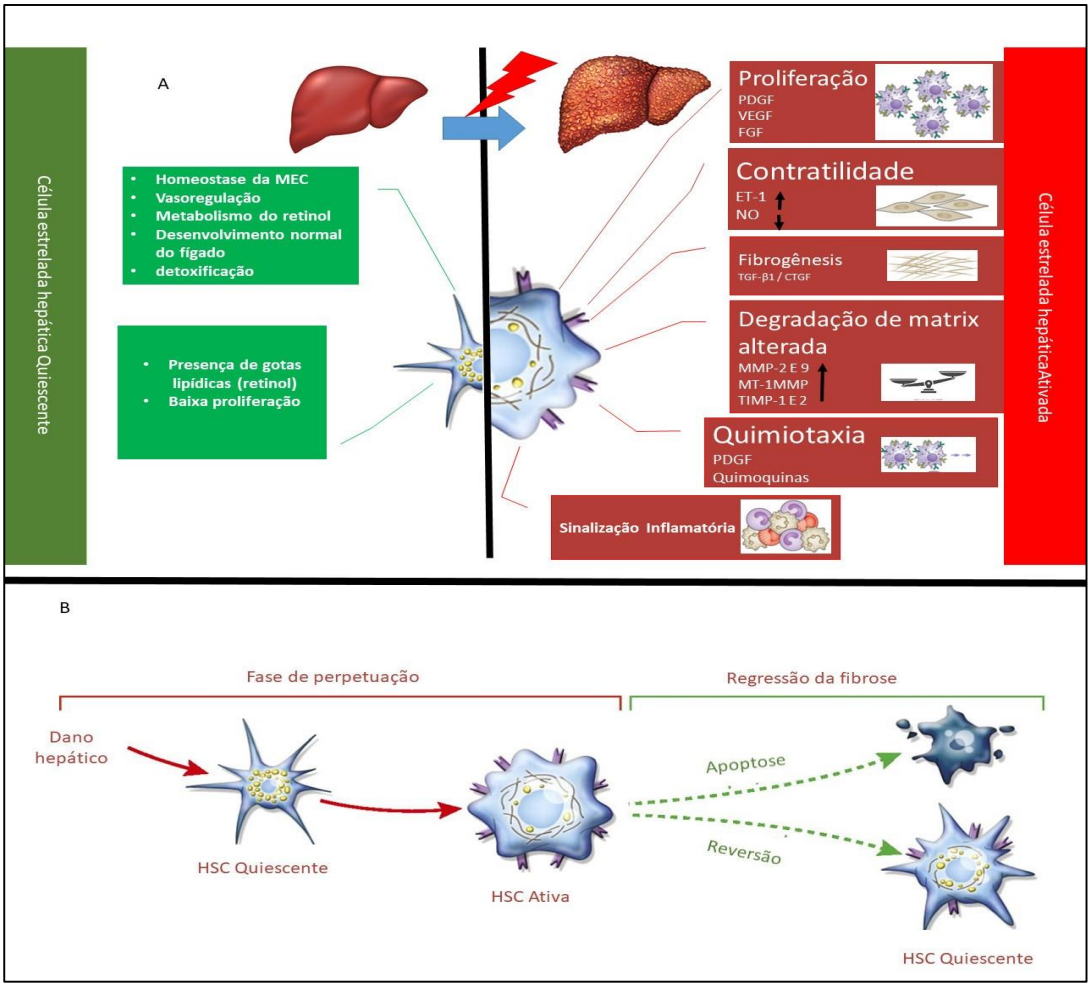

Figura 4: A) Caracteristicas fenotipicas das HSC quiescentes e HSC ativadas. B) Destino das HSC ativadas durante a regressão da fibrose (adaptado de Friedman, 2003, 2012) .

A linhagem GRX apresenta propriedades similares a células do tecido conjuntivo hepático. Possui, portanto, alta taxa de proliferação, capacidade de secretar colágeno tipo I e outras proteínas de matriz extracelular, além de apresentar um padrão de crescimento similar às células musculares lisas, o que lhe confere características morfológicas e bioquímicas de um miofibroblasto: célula conjuntiva encontrada em tecido hepático fibrótico humano. As células GRX podem ser induzidas

à conversão de miofibroblastos para uma célula armazenadora de lipídios semelhante às HSC quiescentes (Borojevic 1987, Monteiro and Borojevic 1987, da Silva, Guimaraes et al. 2003). A transição para o fenótipo lipocítico é acompanhada de diminuição na capacidade de proliferação e de produção de matriz extracelular (Margis and Borojevic 1989, Borojevic, Guaragna et al. 1990). Por outro lado, já foi demonstrado que na presença de mediadores inflamatórios, a GRX responde se transformando em uma célula miofibroblástica ativada (da Silva, Guimaraes et al. 2003, Guimaraes, Franceschi et al. 2007). Devido a essas propriedades, a linhagem celular GRX é um bom modelo de estudo para a compreensão dos processos envolvidos na fibrogênese hepática.

1.4 Resveratrol

O Resveratrol (RSV; 3,5,4′-trihidroxiestilbeno) é uma fitoalexina encontrada em mais de 300 plantas comestíveis, incluindo uvas, mirtilos, framboesas, amoras e amendoins, como um potencial composto de promoção da saúde (Cottart, Nivet-Antoine et al. 2014).

O RSV foi isolado pela primeira vez em 1940, a partir de raízes de heléboro branco (*Veratrum grandiflorum*), e depois em 1963, a partir de *Polygonum cuspidatumraízes*, uma planta usada na medicina tradicional chinesa e japonesa como agente anti-inflamatório e antiplaquetário (Nawaz, Zhou et al. 2017). Este polifenol natural foi detectado em mais de 70 espécies de plantas e também é encontrado em quantidades discretas em vinhos tintos e em vários alimentos humanos (Burns, Yokota et al. 2002).

O acúmulo do RSV em plantas é resultante de um mecanismo de resistência a parasitas e outras condições adversas, como infecção por fungos, radiação UV, substâncias químicas e, em geral, fatores estressantes para a planta. Sua estrutura básica consiste em dois anéis fenólicos ligados entre si por uma ligação dupla de estireno, que forma o 3,5,4′-Tri-hidroxiestilbeno (peso molecular 228,25 g/mol). Estes anéis apresentam ligação dupla que é responsável pelas formas isométricas *cis* e *trans* do resveratrol (figura 5). Vale ressaltar que o isômero *trans* é o mais estável do ponto de vista estérico. Notavelmente, a forma *trans* do RSV é dominante em termos de prevalência e diferentes atividades biológicas são atribuídas a essa forma como, por exemplo, a indução de respostas celulares, a parada do ciclo celular, a diferenciação celular, a apoptose e o efeito antiproliferativo em células cancerígenas (Orallo 2006, Anisimova, Kiselevsky et al. 2011). A molécula de RSV pode sofrer isomerização da forma *trans* para *cis*, que é a sua forma biologicamente ativa quando exposta à irradiação UV e luz visível (Park and Pezzuto 2015).

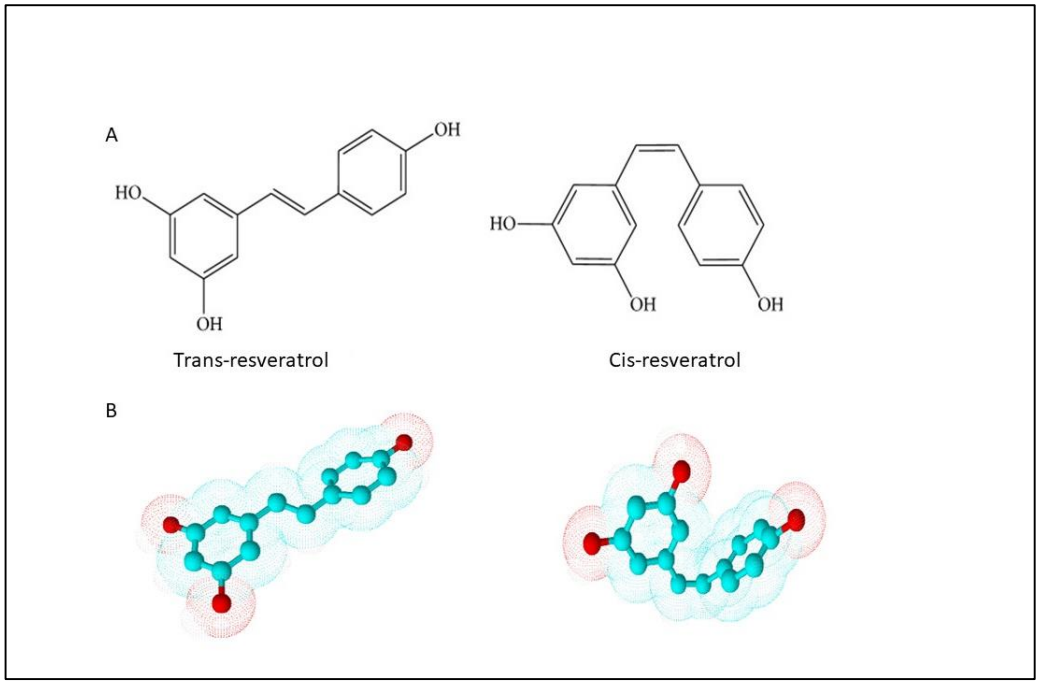

Figura 5: A) Representação da estrutura química do Trans e Cis Resveratrol. B) Representação da estrutura 3D do Trans e Cis Resveratrol (adaptado de Oralo, 2006).

O RSV é amplamente estudado por suas propriedades antioxidantes, anti-inflamatórias e anticarcinogênicas. Além disso, foi proposto que este flavonoide é responsável pelo conhecido “paradoxo francês", originalmente formulado em 1981, onde evidências epidemiológicas francesas demostram que o consumo vinho tinto pode efetivamente reduzir a incidência de doenças cardiovasculares doença (Opie and Lecour 2007). Além disso, foi postulado que o RSV modula as vias de sinalização que limitam a disseminação das células cancerígenas (Tang, Su et al. 2008), protege danos em células nervosas (Jang, Cai et al. 1997, Marambaud, Zhao et al. 2005), ajuda a prevenir o diabetes (Bagul and Banerjee 2015) e atua como um agente antienvelhecimento que melhora os problemas relacionados à idade (Rabassa, Zamora-Ros et al. 2015).

As potenciais funções moleculares do RSV foram descritas pela primeira vez em 2003. Neste trabalho, foi demonstrado que o RSV pode modular a expressão do gene SIRT1, uma desacetilase que afeta a reação de acetilação e a expressão da p53 e de enzimas de reparo de DNA (Howitz, Bitterman et al. 2003). Também já foi demonstrado que em estudos *in vitro*, o RSV atua como modulador de várias proteínas sinalizadoras envolvendo os fatores de transcrição do receptor ativado por proliferador de peroxissomo (PPAR), atuando como antagonista por meio de sua interação direta com as proteínas PPARγ e PPARα (Calleri, Pochetti et al. 2014).

Um estudo do nosso grupo de pesquisa demonstrou que o tratamento com Resveratrol induziu o estado de quiescência de células GRX, modulando a relação PPARγ/SIRT1 (de Souza, Martins et al. 2015). Neste estudo, o tratamento com resveratrol reduziu o acúmulo de gotículas lipídicas em células geneticamente

modificadas que superexpressam o PPARγ (esta linhagem foi batizada como células GRXPγ), bem como, mostrou que a razão PPARγ / SIRT1 desempenha um papel importante na modulação fenotípica das HSC (de Souza, Martins et al. 2015). Também já foi demonstrado que o RSV exerce efeito protetor contra o desenvolvimento de fibrose hepática em ratos tratados com CCl_4 (Chavez, Reyes-Gordillo et al. 2008, Di Pascoli, Divi et al. 2013) e com dimetilnitrosamina (Lee, Shin et al. 2010). O RSV também demonstrou reduzir a área fibrótica e a expressão de αSMA e COL1A1 em fígado de camundongos alimentados com uma dieta rica em gordura quando tratados com lipopolisacarídeo e esse efeito foi atribuído à sua propriedade anti-inflamatória, pois os níveis de mRNA hepático de TNFα e IL-6 diminuíram (Kessoku, Imajo et al. 2016). Outro estudo demonstrou que o RSV pode inibir a via TLR4/NF-κB nas células T-HSC/Cl-6 imortalizada de ratos e nas células LX-2 , ambas linhagens celulares de HSC (Zhang, Sun et al. 2016, Zhang, Sun et al. 2016).

Na linhagem GRX, o RSV promoveu a apoptose via ativação da caspase, e esta situação pode contribuir para um efeito antifibróticos (Meira Martins, Vieira et al. 2015). Bechmann *et al* demonstrou que o RSV amplifica as características de ativação e a capacidade pró-fibrogênica das células LX-2 (Bechmann, Zahn et al. 2009). Esse achado apontou para possibilidade de que, em pacientes considerados obesos e com níveis de ácidos graxos livres (do inglês - *free fatty acids*, FFA) elevados, o tratamento com RSV possa provocar fibrogênese hepática (Bechmann, Zahn et al. 2009). Em contraste, outro estudo demonstrou um efeito anti-fibrogênico do RSV, onde houve o bloqueio da ativação de células t-HSC / Cl-6d, que são células estreladas hepáticas imortalizadas de rato, onde os níveis de NF-κB e da fosforilação de PI3K/Akt foram reduzidos. Neste estudo, os autores concluíram que o RSV foi capaz de inibir a

ativação de HSC e, consequentemente, preveniu a instalação da fibrose hepática (Zhang, Sun et al. 2016). No entanto, ainda não está claro o papel do RSV no processo de ativação HSC, bem como, seu potencial benefício no tratamento de fibrose hepática.

1.5 Bioinformática

A Bioinformática é um dos mais novos campos de pesquisa biológica e interdisciplinar, que usa métodos matemáticos, estatísticos e computacionais para o processamento e análise de dados biológicos (Bellazzi, Diomidous et al. 2011). Nas últimas duas décadas, o rápido crescimento da informação e das tecnologias nas áreas da "genômica" e "ômica" foi imenso e extremamente útil para os cientistas processarem resultados experimentais em grande demanda, tendo em vista que as abordagens tradicionais, de gene por gene na pesquisa, passaram a ser insuficientes para atender ao crescimento da pesquisa biológica e médica (Hyduke, Lewis et al. 2013, Alyass, Turcotte et al. 2015). Consequentemente, as análises dos dados massivos exigiram que as ferramentas computacionais analíticas fossem aprimoradas e que os pesquisadores treinados para sua utilização. O tamanho dos dados gerados por essas metodologias de alto rendimento, juntamente com a necessidade de analisar, de integrar e de interpretar simultaneamente um grande volume de informações de maneira sistêmica, possibilitou uma ampla visão dos fenômenos biológicos (Yan 2014, van Kampen and Moerland 2016).

Do mesmo modo, a utilização de ferramentas computacionais juntamente com a utilização de bancos de dados públicos mostraram-se de suma importância para o rápido desenvolvimento da maioria das ciências da vida, tornando-se uma das bases da biologia moderna (Shumway, Cochrane et al. 2010). De fato, para que a análise de

um fluxo de dados em grande escala fosse possível, houve um aumento nas opções de bancos de dados públicos e privados especializados e na disponibilidade de recursos (*softwares*) para sua exploração, análise e visualização (Coppola, Cianflone et al. 2019). Especificamente, os bancos públicos foram e estão em contínuo desenvolvimento para oferecer suporte ao gerenciamento de tais informações relacionadas a proteômica, genômica, metabolômica para geração de hipóteses orientadas por dados e de descoberta de conhecimento biológico (Chen, Huang et al. 2017). Cabe salientar que, dentre os bancos amplamente utilizados, estão: *Gene Expresion Omnibus* (GEO), é um banco de dados e repositório público internacional que arquiva e distribui gratuitamente a expressão gênica de alto rendimento e outros conjuntos de dados genômicos funcionais (Clough and Barrett 2016); *Database for Annotation, Visualization, and Integrated Discovery* (David), que oferece um vasto banco de dados que fornece um conjunto abrangente de ferramentas de anotação funcional para que os investigadores explorem o significado de eventos biológicos (Dennis, Sherman et al. 2003); *STRING*, é um banco de dados com o objetivo de reunir, avaliar e disseminar informações sobre a associação proteína-proteína de uma maneira abrangente, onde redes de associação proteína-proteína tem uma maior cobertura (cerca de 5090 organismos) e uma ampla gama de ferramentas *on line* amigáveis ao usuário (Szklarczyk, Gable et al. 2019); e *Kyoto Encyclopedia of Genes and Genomes* (KEGG), é um conjunto de bancos de dados e software *on line* associados para compreender e simular comportamentos funcionais das células ou organismo, a partir de suas informações no genoma (Kanehisa, Goto et al. 2004); entre outros.

Dentre as tecnologias atualmente empregadas em bioinformática, estão RNA-Seq, que é uma abordagem recentemente desenvolvida para a criação de perfis de

transcriptoma que utiliza tecnologias de sequenciamento. Isto possibilitou integralizar nossa visão da extensa complexidade das células eucariotas (Manzoni, Kia et al. 2018). A análise destes dados desempenha um papel essencial no entendimento da estrutura e função do genoma, identificando redes genéticas subjacentes aos sistemas celulares, fisiológicos, bioquímicos e biológicos (Mutz, Heilkenbrinker et al. 2013, Manzoni, Kia et al. 2018). Desta maneira, o conjunto destes dados é essencial para interpretar os elementos complexos do genoma que orquestram os fenômenos moleculares em células e tecidos, além de permitir entender o desenvolvimento de patologias (Nagalakshmi, Waern et al. 2010, Martin and Wang 2011), bem como, estabelecer biomarcadores moleculares que possam ser usados para identificar doenças e patógenos.

Os principais objetivos da transcriptômica são: catalogar todas as espécies de transcrição, incluindo mRNAs, RNAs não codificantes e pequenos RNAs; determinar a estrutura transcricional dos genes, em termos de seus locais de partida, extremidades 5 ′ e 3 ′, padrões de emenda e outras modificações pós-transcricionais, quantificar as mudanças nos níveis de expressão de cada transcrição durante o desenvolvimento e sob diferentes condições de interesse (Chen, Wang et al. 2011, McGettigan 2013).

Outro campo amplamente utilizado é a denominada biologia de sistemas, onde é possível analisar o intercâmbio entre os componentes que atuam no sistema biológico. Assim, considera-se que as propriedades emergentes de sistemas biológicos complexos com foco nas propriedades topológicas ou nas distribuição de fluxo de redes em grande escala, ou no comportamento dinâmico de seus componentes moleculares (por exemplo, genes, proteínas, metabólitos) (Panagiotou

and Nielsen 2009, Klassen, Faccio et al. 2017). Deste modo, estas ferramentas e as novas técnicas de simulação computacional permitiram o estudo de redes em grande escala e do impacto de sua regulação/desregulação para saúde (Eslam and George 2020). A análise de rede, que é um ramo da biologia computacional, oferece abordagens para tomar diferentes tipos de elementos, chamados de 'nodos', e as suas relações ('*edges*') entre estes elementos/nodos para gerar hipóteses sobre a rede de interações resultante (Chautard, Thierry-Mieg et al. 2009). Este tipo de análise possibilita estimar padrões complexos de relacionamento e a estrutura da rede construída pode ser analisada para revelar os principais recursos relacionados ao conjunto de dados sobre genes e proteínas individuais, que precisam ser interpretados baseados no contexto celular e no conhecimento biológico existente (Ram, Mendelsohn et al. 2012, Eslam and George 2020). Por conseguinte, modelos de rede biológica, incluindo redes metabólicas, regulatórias da transcrição, interação proteína-proteína, sinalização e co-expressão, formam um arcabouço para estudar as vias biológicas, como as que operam no fígado, em conexão com o desenvolvimento da doença de uma maneira sistemática (Ram, Mendelsohn et al. 2012). Neste aspecto, a análise de expressão diferencial é amplamente utilizada. Assim, compara-se o valor médio de expressão de genes, entre os genes das amostras em estudo, para identificar genes expressos de forma significativa por meio de testes estatísticos (de la Fuente 2010). Para este tipo de análise, a utilização de software como R e MATLAB são amplamente utilizados. Especificamente, o programa R proporciona uma grande gama de pacotes, que são adicionados para acrescentar novas funcionalidades ao programa.

1.6 Bioinformática, Fibrose hepática e Células Estreladas Hepáticas

Naturalmente, a utilização de técnicas computacionais está envolvida em muitos estudos sobre fibrose hepática nos últimos anos. Dado que o fígado é o maior órgão e um importante centro metabólico do corpo, explorar a assinatura metabólica da fibrose hepática é uma promessa para a descoberta de novos marcadores e alvos terapêuticos (Bataller and Brenner 2005, Friedman 2007). Neste aspecto, o emprego destas técnicas tem sido amplamente utilizado no estudo de vias metabólicas presentes que atuam em processos celulares responsáveis pela fibrose e da ação de potenciais alternativas farmacológicas nestes processos (Chang and Yang 2019). Da mesma forma, a análise de expressão diferencial de genes tem, como objetivo, identificar os genes envolvidos nos processos responsáveis tanto pela identificação de químicos e fármacos quanto na modulação de diversos tecidos e células responsáveis pela fibrose hepática (Shangguan, Tan et al. 2015). Por exemplo, Liang *et al* estabeleceu uma rede incluindo dados de interação, utilizando a técnica de transcriptômica e a análise integrada de redes de proteína-proteína para elucidar o mecanismo molecular da apoptose induzida por taurina em HSC, através da regulação positiva de TGFB1 e da ativação da via p38 MAPK-JNK-Caspase 9/8/3 (Liang, Liang et al. 2019). Outro estudo analisou uma longa rede de expressão não codificante (lncRNA) -mRNA e os possíveis papéis destes em HSC primárias de rato durante o processo de ativação, onde a lncRNA-mRNA desregulada indica papéis potencialmente críticos para NONRATT013819.2 e Lox na remodelação de MEC durante a ativação de HSC (Guo, Xiao et al. 2017). Cabe ressaltar que as análises, tanto de fibrose hepática quanto na modulação fenotípica das HSC, são utilizadas várias abordagens de estudo, desde técnicas *in vivo* e *in vitr*o, até técnicas *in sílico,*

onde recursos computacionais disponíveis e modelos matemáticos/ estatísticos são utilizados.

2 JUSTIFICATIVA

A fibrose hepática é uma resposta de cicatrização de lesões hepáticas crônicas, que pode levar à cirrose e à insuficiência hepática, podendo culminar em hepatocarcinoma e morte. Desta forma, ressalta-se a importância de pesquisas para encontrar tratamentos alternativos para o tratamento da fibrose hepática. Considerando os efeitos que o RSV tem demonstrado ao longo de anos de pesquisa do nosso grupo, torna-se pertinente investigar seus efeitos nos parâmetros de ativação/quiescência das HSC (linhagem GRX), bem como seus efeitos na liberação de citocinas inflamatórias por estas células. Com as ferramentas de bioinformática, uma metodologia de estudo mais recente, foi utilizada analisar os principais genes alvos conhecidos do RSV usando bancos de dados públicos, fazendo um levantamento sobre seu papel na modulação fenotípica das HSC. Os entendimentos destes eventos bem como de suas inter-relações são, sem dúvida, importantes para a caracterização e para a busca da resolução da fibrose hepática.

3 OBJETIVOS

3.1 Objetivos gerais

O objetivo geral deste trabalho foi avaliar a influência do resveratrol na manutenção do estado de ativação, na promoção de quiescência e na sinalização inflamatória das células estreladas hepáticas. Os possíveis genes alvo do resveratrol assim como foram determinados por meio de técnicas integradas de bioinformática.

3.2 Objetivos específicos

1 – Quantificar marcadores proteicos clássicos de ativação das HSC, aSMA, colágeno tipo I, GFAP, em células da linhagem GRX tratadas com resveratrol.

2- Analisar as alterações morfológicas e a reestruturação do citoesqueleto em células da linhagem GRX tratadas com resveratrol.

3- Analisar capacidade de migração das células da linhagem GRX tratadas com resveratrol.

4- Comparar a habilidade de contração celular em células GRX tratadas com resveratrol.

5 - Quantificar em todos os tratamentos a secreção de interleucinas e dos fatores TNF-α, interleucina-6 e interleucina- 10.

6 - Identificar os potenciais genes alvos do resveratrol em bancos de dados públicos utilizando ferramentas de bioinformática.

7- Analisar o papel dos potenciais genes alvos do resveratrol na modulação fenotípica das células estreladas hepáticas.

8-Analisar por meio de microarranjos e por ferramentas de bioinformática o padrão de expressão gênica das HSC quiescentes vs. ativadas.

9- Analisar as principais vias metabólicas dos genes das HSC quiescentes vs. ativadas.

10 – Construir e analisar as redes de interação proteina-proteina dos genes diferencialmente expressos.

Parte II

CAPÍTULO 1

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Resveratrol increases the activation markers and changes the release of inflammatory cytokines of hepatic stellate cells

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Abstract
The phytoalexin Resveratrol (3,5,4′-trihydroxystilbene; RSV) has been related to numerous beneficial effects on health by its cytoprotection and chemoprevention activities. Liver fibrosis is characterized by the extracellular matrix accumulation after hepatic injury and can lead to cirrhosis. Hepatic stellate cells (HSC) play a crucial role during fibrogenesis and liver wound healing by changing their quiescent phenotype to an activated phenotype for protecting healthy areas from damaged areas. Strategies on promoting the activated HSC death, the quiescence return or the cellular activation stimuli decrease play an important role on reducing liver fibrosis. Here, we evaluated the RSV effects on some markers of activation in GRX, an HSC model. We further evaluated the RSV influence in the ability of GRX on releasing inflammatory mediators. RSV at 1 and 10 µM did not alter the protein content of α-SMA, collagen I and GFAP; but 50 µM increased the content of these activation-related proteins. Also, RSV did not change the myofibroblast-like morphology of GRX. Interestingly, RSV at 10 and 50 µM decreased the GRX migration and collagen-I gel contraction. Finally, we showed that RSV triggered the increase in the TNF-α and IL-10 content in culture media of GRX while the opposite occurred for the IL-6 content. Altogether, these results suggested that RSV did not decrease the activation state of GRX and oppositely, triggered a pro-activation effect at the 50 µM concentration. However, despite the increase of TNF- α in culture media, these results on IL-6 and IL-10 secretion were in accordance with the anti-inflammatory role of RSV in our model.

Keywords Hepatic stellate cells · Liver fibrosis · Liver wound healing · Resveratrol

Introduction

Resveratrol (3,5,4′-trihydroxystilbene; RSV) is a phytoalexin produced by several plant species, such as peanuts and grapes, in response to pathogenic infection and environmental stresses. This molecule is present at relevant concentrations in red wine and has been related to numerous beneficial effects on health by its cytoprotection and chemoprevention activities, which has been largely associated with its anti-inflammatory and anti-oxidant effects. Paradoxically, RSV can also exert cytotoxicity through inducing cell death and cell growth inhibition, two positive effects for treating several pathological conditions such as cancer [1, 2].

Liver fibrosis is a dynamic process characterized by the accumulation of extracellular matrix resulting from hepatic injury, including those caused by a viral infection, alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH) and hepatic steatosis. It is a consensus that liver fibrogenesis

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can be reversed if the cause of injury is ceased. However, the unchecked chronic liver injury and fibrogenesis can lead to cirrhosis, which compromises the hepatic architecture leading to abnormal blood flow and eventually to portal hypertension. In addition, liver cirrhosis may lead to the onset of complications, such as hepatic encephalopathy and variceal haemorrhage, which may increase the chances of hepatocellular carcinoma (HCC) development and, thus, the mortality risk [3, 4].

Hepatic stellate cells (HSC) are known to store lipid droplets enriched in retinyl ester in their cytoplasm, a condition that characterizes their quiescent phenotype. As one of their physiological features, HSC can differentiate into an activated phenotype in response to paracrine stimulation from damaged hepatocytes after liver injury. At this condition, HSC loses their lipid droplets and becomes fibrogenic myofibroblast-like cells, playing an important role in liver wound healing through protecting healthy areas from damaged areas. Nonetheless, continuous damage to the liver results in a chronic inflammatory response in which hepatic environment may not recover its homeostatic balance. In this context, increased production/activity of cytokines may be critical for both autocrine and paracrine perpetuation of HSC activation, which contributes to the excessive extracellular matrix accumulation that leads to liver fibrosis. In this way, strategies that promote the activated HSC death, the quiescence return or the cellular activation stimuli decrease play an important role on treating chronic liver injuries, focusing in the liver fibrosis reduction [5–7].

Along the past years, our research group has been studying the effects of RSV treatment in the murine cell line GRX, an activated HSC model [8]. GRX cells have been an excellent tool for studying the extrinsic and intrinsic factors that could trigger or prevent liver fibrosis since these cells can be induced to display the HSC quiescent-like or a more activated-like phenotype [9–13]. We already found that RSV treatment (0.1–50 μM) compromised the GRX cell viability through inhibiting cell cycle at the S-phase, impaired mitochondria and induced apoptosis, especially in the cell group that received the highest dose, where the cell population was drastically reduced. However, it was interesting that these effects were attenuated by the concomitant induction of mitochondrial biogenesis and autophagy, two survival mechanism against cellular environmental toxicity, which culminated in the GRX resistance to the cytotoxic effects of RSV [14–16]. Further, we found that 0.1 μM of RSV was not able to restore the GRX capacity of storing lipid droplets. On the contrary, our results suggested that RSV could play a SIRT1-mediated lipolysis in GRX stimulated to store lipid droplets by Retinol treatment or by PPARγ super expression [17].

RSV treatment showed positive effects on compromising viability or reducing the number of activated HSC especially at the highest concentration (50 μM). On the other hand, RSV compromised the ability of GRX on storing lipid droplets, a characteristic of quiescent HSC. Here, we seek for evaluating the effects of RSV towards HSC activation by measuring some molecular markers and cell migration after wound induction in GRX cell culture. We further evaluated the effects of RSV in the HSC ability on releasing TNF-α, IL-6 and IL-10 in the culture media, considering the importance of these cytokines during liver fibrogenesis.

Material and methods

Cell culture

The GRX cell line was obtained from the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). For most experiments, $3 \times 10^4/cm^2$ cells were seeded in 24-well culture plates (Nunc, Roskilde, Denmark). For cell migration evaluation, $1.5 \times 10^4/cm^2$ cells were seeded in 96-well culture plates (Nunc, Roskilde, Denmark). During culture, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (Cultilab, Campinas, SP, Brazil) and 2 g/L HEPES buffer (pH 7.4) in a humidified atmosphere containing 5% CO_2 at 37 °C.

Resveratrol treatment

Resveratrol (Sigma Inc., St. Louis, MO, USA) was dissolved in 20 μL of ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and sequentially diluted in DMEM to a final concentration of 1, 10 and 50 μM just before use. After reaching confluence, cells were treated for 24 h. Vehicle-treated cells were considered experimental control.

Analysis of HSC activation markers by flow cytometry

The cellular protein content for glial fibrillary acidic protein (GFAP), collagen I and smooth muscle actin-α (SMA-α) in GRX treated with RSV was measured by flow cytometry. Briefly, after 24-h treatment, cells were harvest by trypsin/EDTA (Sigma Inc.) and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 15 min. Sequentially, cells were overnight incubated with the primary antibodies (GFAP, n.34001, from Cell Signalling, Danvers, MA, USA; collagen I, n.8784, from Santa Cruz Biotechnology, Dallas, TX, USA; SMA-α, n.A5228, from Sigma Inc.) diluted in PBS with 5% of albumin (1:500). Then, cells were incubated with adequate secondary antibodies (1:1000, diluted in PBS with 5% of albumin) for 2 h at room temperature: GFAP and SMA-α-labelled cells were exposed to anti-mouse

AlexaFluor 488 (n.A11001, from Invitrogen) while collagen I-labelled cells were exposed to anti-goat AlexaFluor 647 (n.A21447, from Invitrogen). Cells incubated only with respective fluorescence secondary antibodies were used as negative controls. A total of 20,000 events were acquired by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at FL-1 (green fluorescence) and FL-4 (red fluorescence) channels. All data analyses were performed with FCS Express 4 software (De Novo, Software, Ontario, Canada). Results were expressed as fluorescence units [Control = 1].

Analysis of HSC morphology by confocal microscopy

For analysing the cytoskeleton morphology, GRX was stained with tetramethyl-rhodamine isothiocyanate–phalloidin (Invitrogen), which specifically binds to F-actin with high affinity. Briefly, cells were cultured under coverslip, fixed in 4% paraformaldehyde for 15 min at 4 °C and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Filamentous actin was stained in accordance to the manufacturer's instructions. Images were collected using Olympus FV1000 laser-scanning confocal microscope. Ten single confocal sections of 0.7 µM were taken parallel to the bottom plates (*xy* sections) with a × 60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super apochromat, UPLSAPO60XO). Images from six random fields were acquired and deconvolved using the interactive 3D plugin of ImageJ software (https://rsb.info.nih.gov/ij).

Analysis of HSC-induced contraction of collagen I gel

In order to evaluate the RSV effects in the HSC ability of contracting ECM, a characteristic of activated cells, a ready-to-use storable gel of collagen I was prepared after extracting it from rat tail tendon as previously described [18]. Animals were obtained from the Center for Experimental Biological Models at *Pontifícia Universidade Católica do Rio Grande do Sul* (PUCRS) and kept in a controlled temperature environment (24 ± 2 °C), light/dark cycle of 12 h, with free access to water and food. The experimental protocol was approved by the Ethics Research Committee of PUCRS.

Collagen I gels (constituted by 125 µl of 4 × DMEM and 125 µl of 4 mg/mL Rat Tail Tendon extracted collagen I) were impregnated with 10^5 cells resuspended in 250 µl of PBS and added into a 24-well plate for polymerising at 37 °C during 1 h. Then, collagen I gels and impregnated cells were detached and suspended in culture (control) and treatment (1, 10 and 50 µM of RSV) media. Images were acquired in a gel documenter (L-Pix, Loccus, Cotia, SP, Brazil) after 24 h of treatment, and the surface area for each gel was determined as percentage of well area using ImageJ software (https://rsb.info.nih.gov/ij/), a public domain Java image processing software. Cell treatment with *N*-acetylcysteine (NAC) at 400 µg/mL was used as a positive control [19]. Results were expressed as area of gel, considering control as 100%.

Analysis of HSC migration by in vitro scratch assay

The effect of RSV in the migration capacity of GRX was evaluated by the in vitro scratch assay as previously described [20]. Briefly, after 24-h treatment, a circular gap was created with a 200-µL pipette tip at cell-confluent monolayer. Then, 24 images for each group were acquired in the SpectraMax i3 Multi-Mode Platform (Molecular Devices, Sunnyvale, CA, USA) at 0, 6, 12 and 24 h. Wound area at the aforementioned times was measured using ImageJ software. Results were expressed as the percentage of cell migration which represents the wound area reduction (wound closure).

Analysis of HSC inflammatory release by ELISA assay

After 24-h treatment, the interleukine-6 and interleukine-10 concentrations were quantified in cell culture media using Quantikine ELISA Kit and protocol (R&D Systems, Minneapolis, MN, USA). Tumour necrosis factor-α concentration in the culture medium was determined using Sigma ELISA Kit (Sigma Inc.), accordingly to the manufacturer's protocol. Optical density was collected in a microplate fluorimeter reader (M5, Molecular Devices, USA). For allowing a more precise estimate for the RSV effects on cytokine releasing by GRX, cell quantity had to be considered. Results were then normalized by protein content [21] at the end of RSV treatments and were expressed as pg/µg.

Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Experiments were repeated at least three times ($n = 3$). One-way ANOVA was used to analyse the effect of RSV treatment, and Tukey *post-hoc* was performed. Results were considered statistically different when the p values were less than 0.05.

Results

Resveratrol induces an increase in the protein markers of activation in HSC

HSC have well-known molecular markers for activation and myofibroblast differentiation and among them, the increase in the protein content of GFAP, collagen I and SMA-α [3, 5, 22–24]. Thus, we sought to evaluate the content of these

proteins in GRX by flow cytometry. Treatment with 1 and 10 μM of RSV did not alter the intracellular GFAP, collagen I and SMA-α protein quantity; however, 50 μM of RSV triggered an increase in the protein content of these molecular markers of activation in GRX (Fig. 1).

Resveratrol does not alter HSC cytoskeleton morphology, but the highest concentration decreases cells ability of contracting collagen I gel

The increase of cell contractibility, characterized by the F-Actin cytoskeleton rearrangement in stress fibres, is an important feature of activated HSC [25, 26]. Thus, RSV-treated cells were stained with tetramethyl-rhodamine isothiocyanate–phalloidin for evaluating cell cytoskeleton. No significant changes were observed since all groups were presented with cells with stress fibres and a myofibroblastic-like morphology with elongated cytoplasm (Fig. 2a). However, the collagen I gels containing cells treated with 50 μM of RSV were significantly less contracted (Fig. 2b).

Resveratrol induces a decrease in the HSC migration

The increase in the cell migration ability is also a feature of activated HSC [27]. Thus, we also evaluate GRX capacity on migrating after creating a circular gap in cultured cell at monolayer. Interestingly, after 6 and 12 h of wound creation, cells treated with 10 and 50 μM of RSV presented a decreased rate of migration. At 24 h after wound creation, only cells treated with 50 μM of RSV remained with a decreased rate of migration (Fig. 3).

Resveratrol alters the HSC capacity of releasing tumour necrosis factor-α, interleukin-6 and interleukin-10 in cellular medium

Numerous cytokines, which may be pro- or anti-fibrogenic, have been shown to play a major role in wound-healing response during liver diseases [3, 5–7]. Therefore, the largely discussed anti-inflammatory property RSV [1, 28–30] may interfere on cytokines signalling among cultured HSC. Thus, we evaluated the RSV effects in the GRX ability on releasing TNF-α, IL-6 and IL-10; three important cytokines that are involved in liver fibrogenesis [5, 22, 31, 32]. All concentrations of RSV were able to increase the release of TNF-α in culture medium by GRX (Fig. 3a) while treatment with 10 and 50 μM triggered a similar effect for IL-10 releasing (Fig. 3c). Oppositely, all concentrations of RSV triggered the decrease on IL-6 releasing by GRX in culture media (Fig. 3b).

Discussion

The phytoalexin resveratrol (RSV) has attracted a lot of researchers' attention for being a nutraceutical compound with a large pharmacological potential for clinical treating of many diseases. In this regard, the potential health-promoting properties of RSV have been associated to its pleiotropic-like effects, which are a consequence of its interaction with a large number of signalling pathways that covers a broad range of pathologies including cancer, metabolic syndrome, cardiovascular diseases, neurodegenerative disorders, ageing and inflammation [1, 28, 33–35].

Understanding liver fibrosis focuses primarily on events that lead to activation and proliferation of HSC, which consists of two major phases: initiation and perpetuation. Under liver inflammatory condition, the paracrine stimuli from neighbouring cells—namely injured hepatocytes, endothelial cells, Kupffer cells and platelets—initiate HSC activation. The pathways for perpetuating the activated HSC phenotype include the acquisition of new functions such as proliferation, release of pro-inflammatory cytokines, matrix rearrangement and fibrogenesis. The HSC activation may substantially contribute for the maintenance of liver cirrhosis that can culminate in hepatocarcinoma or liver failure. Thus, the search for treating chronic liver disease, including advanced cirrhosis, shall be focusing in the liver fibrosis regression by controlling the fibrotic activity of activated HSC through inducing these cells to quiescence or apoptosis [3–7].

Recent studies have demonstrated the preventive and therapeutic role of RSV for many liver disorders. Among these beneficial effects, RSV was able (1) to provide liver protection against chemical, cholestatic and alcohol injury; (2) to improve glucose metabolism and lipid profile, thus decreasing liver fibrosis and steatosis; (3) to increase the survival period after liver transplantation; and (4) to decrease fat deposition, necrosis and apoptosis in hepatocytes after liver ischemia–reperfusion (I/R) injury. When focusing on HSC metabolism, previous studies had shown the RSV effects on promoting the reduction of α-SMA protein content [28, 29]. In light of the huge number of studies pointing the beneficial effects of RSV for treating numerous pathologies including liver diseases, our research group has been studying the effects of this phytoalexin in GRX cell line, seeking for evaluating its treatment effects focusing on activated HSC.

RSV was indeed cytotoxic to GRX, but these effects seemed to be dose-dependent, being attenuated along time of cell treatment. Furthermore, this phytoalexin was not able to restore the capacity of GRX cells on storing lipid droplets. Oppositely, RSV treatment promoted lipolysis in quiescent-like cells [14–17], which is an event that

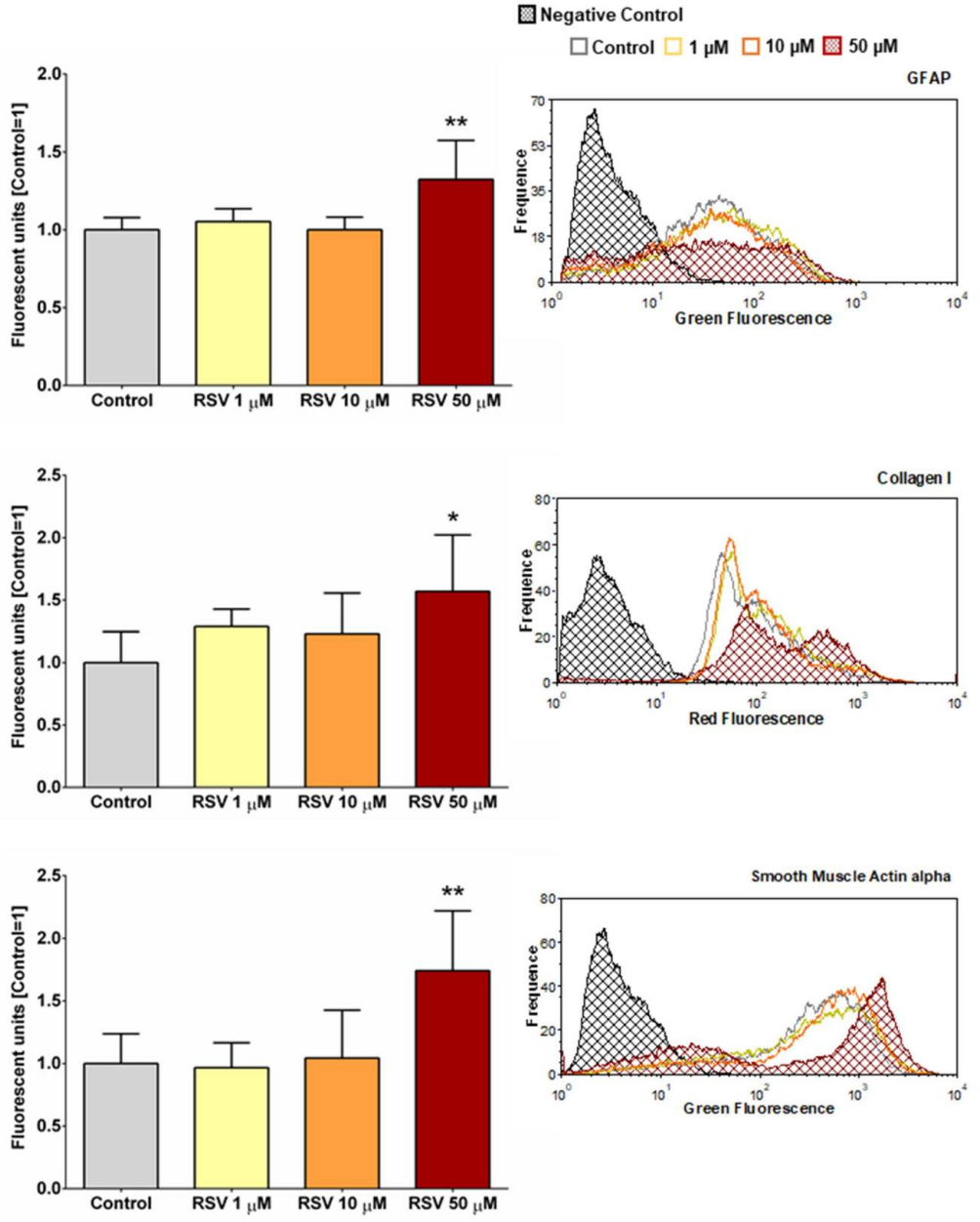

Fig. 1 RSV at 1 and 10 µM concentrations did not alter the protein content of GFAP, collagen I, and SMA-α; however, the highest concentration, 50 µM, triggered an increase in these molecular markers of activation in GRX ($n = 3$, mean ± SDM, $p < 0.05$)

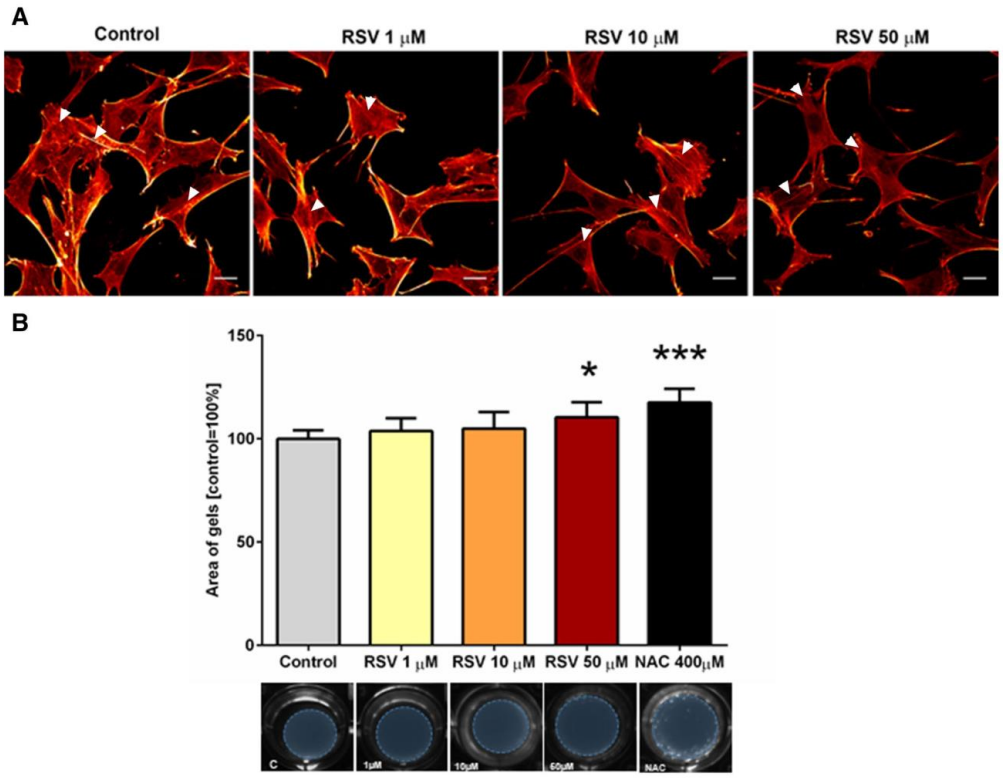

Fig. 2 **a** No significant changes were observed in GRX cytoskeleton since all-treated groups presented cells with stress fibres (white arrows) and a myofibroblastic-like morphology with elongated cytoplasm (images were pseudo-coloured in Red Hot by ImageJ; Scale bar: 10 μm); **b** Collagen I gels containing cells treated with RSV at 50 μM were significantly less contracted. *N*-acetylcysteine (400 μg/mL) was used as a positive control

characterizes the early steps of HSC activation [3–5]. Thus, we sought to first evaluate some parameters of HSC activation in response to a 24-h treatment with RSV at 1, 10 and 50 μM. We found that RSV at lowest concentrations (1 and 10 μM) did not alter the protein content of α-SMA, collagen I and GFAP. However, treatment with 50 μM of RSV induced an increase of content for these activation-related proteins. The increase of contractibility in activated HSC is an important feature that contributes to the intrahepatic resistance and portal hypertension, which is responsible for the morbidity in liver cirrhosis. In this situation, activated HSC displays morphological changes in their cytoskeleton characterized by the presence of F-actin stress fibre [25, 26]. In this way, RSV was not able to change the myofibroblast-like morphology of GRX, which remained displaying elongated cytoplasm characterized by the presence of stress fibres. Altogether, these results suggested that RSV treatment did not decrease the activation state of GRX and, oppositely, triggered a pro-activation effect at the 50 μM concentration.

Another remarkable feature of activated HSC is their ability to migrate towards damaged areas after liver injury, a chemotactic effect that is important to wound healing and hepatic tissue remodelling. However, an increased migration of activated HSC could exacerbate the fibrotic progression, worsening organ dysfunction [3, 27]. Cells treated with 10 μM of RSV presented a decreased rate of cell migration after 6 and 12 h from wounding while this effect was observed in cells treated with 50 μM of RSV at all times of wounding evaluation. In addition, it was also notable that 50 μM of RSV apparently decreased cells ability of contracting collagen I gels. At first sight, all results regarding the RSV effects in GRX activation, contraction ability and migration were surprising and seemed to be contradictory,

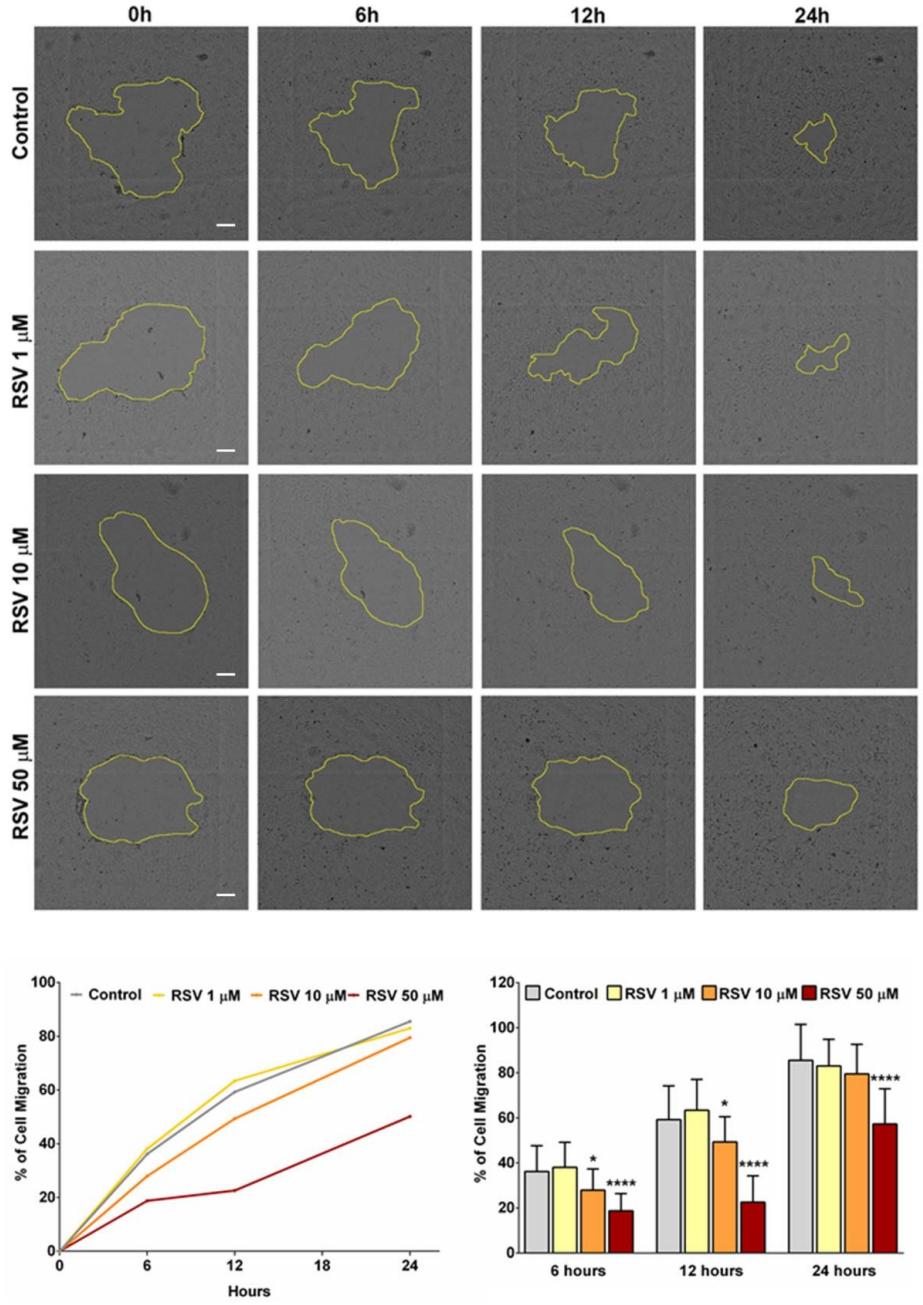

Fig. 3 Cells treated with 10 μM of RSV presented a decreased rate of cell migration after 6 and 12 h from wounding while this effect was observed in cells treated with 50 μM of RSV at all times of wounding evaluation ($n = 3$, mean ± SDM, $p < 0.05$, Scale bar: 200 μm)

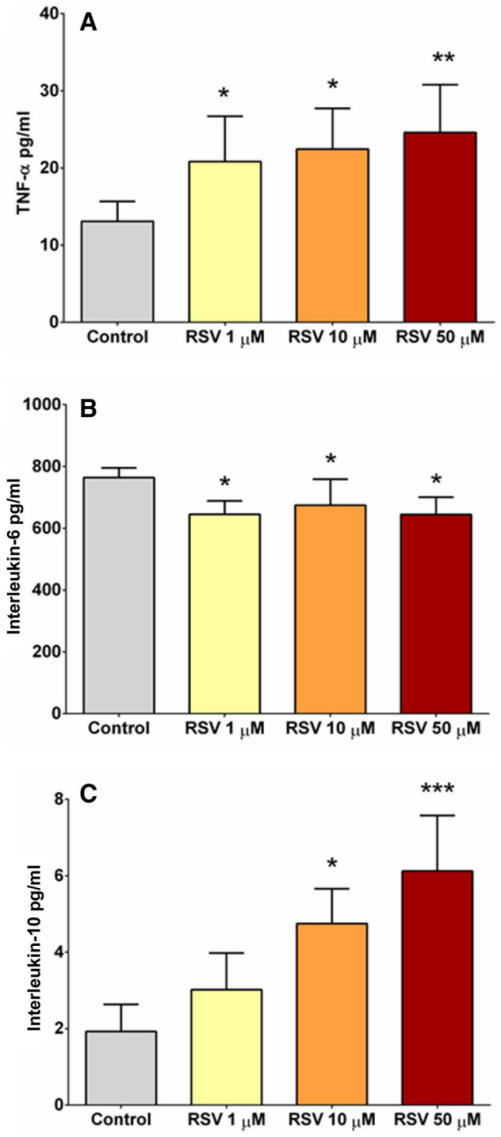

Fig. 4 **a** All concentrations of RSV triggered the release of TNF-α in culture medium by GRX; **b** An opposite effect was observed regarding the release of IL-6; **c** RSV at 10 and 50 μM concentrations triggered the release of IL-10 in culture medium by GRX ($n = 3$, mean ± SDM, $p < 0.05$)

especially on those cells that were treated with the highest concentration. However, it is necessary to consider that RSV was cytotoxic to our model [14–16]. Once the HSC is activated in response to stress mediators [3–5], and here RSV appears to display a stressor behaviour, it seems to be a plausible hypothesis that GRX cells respond to the RSV cytotoxicity through remaining at their activation state or, in the scenario by which this phytoalexin is remarkably toxic, through increasing their activation state. Furthermore, cytotoxicity and low cell migration use to be correlated events [36–38], which can make sense to explain the RSV effects on impairing GRX migration regardless the fact of these cells remaining at an activated—or more activated—phenotype. In the same way, the reduction of cell population due to the cytotoxicity of RSV at 50 μM after 24-h treatment, as previously demonstrated [14], may be related to the smaller contraction of collagen I gel in this group.

Most of the new cellular functions of activated HSC are indeed sustained by an autocrine loop characterized by the enhancement of cell response to several mediators through both the upregulation of their membrane receptors and the enhancement of intracellular signalling [3–5]. Also, HSC-mediated inflammatory signalling may influence the function of hepatocytes and sinusoidal cells, and may favour the repair of injured tissue through promoting the restoration of hepatic homeostasis [3–5, 22]. Thus, we assume that the largely discussed anti-inflammatory property of RSV [1, 2, 28] would interfere on cytokines signalling mediated by HSC. Here, we showed that RSV was able to increase the release of TNF-α and IL-10 by GRX in culture media while the opposite occurred for the IL-6 releasing. Considering these results and since our model represents a restricted population of liver cells, the presence of TNF- α and IL-10 in culture media could influence the GRX itself metabolism.

Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are important pro-inflammatory and pro-fibrogenic mediators that participate in the HSC activation during initiation phase. Curiously, some studies have also suggested that TNF-α can reduce liver fibrogenesis by inducing activated HSC to synthesize less collagen I during their perpetuation phase. Also, there are evidences that TNF-α mediates a pro-apoptotic pathway in which the mitochondrial potential decreases while reactive oxygen species (ROS) and caspase cascade act as downstream mediators, and this scenario is in accordance to the RSV effects on GRX, which were found in our previous studies. Similarly, IL-6 is thought to exert beneficial effects during liver chronic diseases through playing an important role for inducing the hepatocytes regeneration.

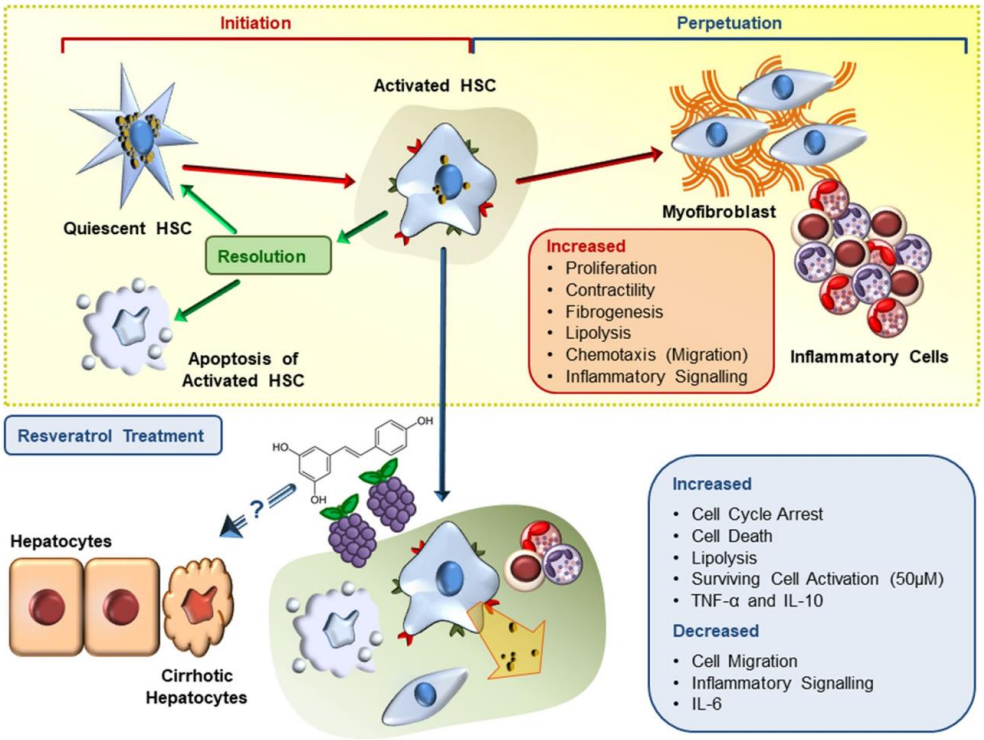

Fig. 5 Treatment of chronic liver disease, including advanced cirrhosis, shall be focused in the liver fibrosis regression by controlling the fibrotic activity of activated HSC through inducing these cells to quiescence or apoptosis. Our previous findings showed that RSV treatment induced cell cycle arrest, cell death, and lipolysis in GRX cell line, which is an activated HSC model. Here we found that RSV at 1–10 μM did not decrease in HSC activation state; oppositely, the highest concentration induced an increase of activation markers (GFAP, collagen I, and SMA-α). However, RSV treatment decreased activated HSC migration and triggered an anti-inflammatory effect. Further studies are needed to elucidate the RSV effect on other liver cells, especially healthy or cirrhotic-injured hepatocytes

These facts reveal that both TNF-α and IL-6 have, indeed, a pleiotropic function during the HSC activation process [32, 39–43]. Interleukin-10 (IL-10) has been regarded as one of the most important anti-inflammatory cytokines even in the presence of higher levels of pro-inflammatory cytokines [1, 2, 29, 34, 44]. Also, IL-10 may act on preventing an excessive liver fibrogenesis or an inappropriate inflammatory response through inducing activated HSC to apoptosis [45–48]. Altogether, considering that GRX is an activated HSC model, these results on the TNF- α, IL-6 and IL-10 media content may indicate an important role of RSV in our model, which may be helpful for controlling HSC activity during liver fibrosis (Fig. 4).

Several studies have demonstrated that RSV can exert contradictory effects depending on its concentration, time of treatment or model of study. Indeed, RSV studies in cell culture models have demonstrated that low concentrations of this molecule increase proliferation while high concentrations impair cell growth [1, 2, 28, 49]. Until now, we found several relevant effects on treating activated HSC with RSV (Fig. 5). However, it is relevant to point that, during liver fibrosis associated to cirrhosis, at the same time by which is expected to control the proliferation of activated HSC or to induce apoptosis or quiescence of activated HSC, the hepatocyte regeneration or survival against damage stimuli must be sought [3, 6, 7, 50]. The RSV-mediated HSC release of TNF-α and IL-10, and the decrease of IL-6 release, may influence not only HSC itself but also hepatocytes. Thus, it is undoubtedly relevant to consider the effects of RSV for other liver cells on considering this phytoalexin for treating liver fibrosis during chronic hepatic diseases and cirrhosis. In this way, more studies focusing on the HSC relationship with

other liver cells, especially hepatocytes, shall be conduct for a better understanding of the RSV effects to liver as whole.

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Author contributions Wrote the paper: CMO and LAMM. Establishment of GRX cell line: RB. Collected and analysed data: CMO, LAMM, ASC, KSM, BruPC, MQV, BarPC. Supervision and Contribution to the text writing: JRO and FCRG.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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CAPÍTULO 2

*__Manuscrito de Bioinformática atualmente em desenvolvimento.__

INTEGRED BIOINFORMATICS ANALISYS TO IDENTIFY THE THERAPEUTC TARGETS OF RESVERATROL IN HEPATIC STELLATE CELLS

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Abstract

Liver fibrosis is a public health problem that is characterized by the excessive accumulation of extracellular matrix proteins. The hepatic stellate cells (HSC) are the major target for the treatment of liver fibrosis. Resveratrol (RSV) has been shown to have biological effects against different diseases, among them, liver fibrosis. Thus, we explore the possible direct target proteins (DTPs) of RSV and their potential roles in the phenotypic modulation of HSC by using integrated bioinformatics resources and public databases. Firstly, it was identified 26 DTPs in the DRUGBANK database. Then, protein-protein interaction (PPI) network and Reactome pathways was analyzed. Further, scientific papers on the target genes of RSV were searched in the PUBMED database. We found 26 possible DTPs for RSV. We find that only 7 DTPs have already been associated with studies on liver fibrosis in the PUBMED database. In summary, we showed that studies on the possible DTPs of RSV are still need for clarifiying their role for modulating the HSC phenotype.

Keywords: Resveratrol, Hepatic stellate cells, liver fibrosis, bioinformatics, public databases, Drug Bank.

1 Introduction

Liver fibrosis can be induced by chronic liver injuries and is a problem worldwide. These hepatic injuries include those caused by viral infection, alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), and hepatic steatosis (Puche, Saiman et al. 2013). In normal condition, liver regenerates after injury; however, when liver suffers chronic damage and inflammation, it cannot normally regenerate. The exacerbated wound healing response causes the alteration and the deposit of extracellular matrix (ECM), causing tissue scarring (Bataller and Brenner 2005). Progressive liver fibrosis results in cirrhosis, where liver cells cannot function properly due to the formation of fibrous scar and regenerative nodules that causes the decreased blood supply to the liver (Friedman and Hao 2017). There is still no effective therapy available for this condition, except for the removal of underlying etiology or liver transplantation (Zoubek, Trautwein et al. 2017).

The responsible for liver fibrosis are the hepatic stellate cells (HSC), which are responsible for depositing the type I collagen, the main feature of liver fibrosis (Puche, Saiman et al. 2013). HSC display two phenotypes. In the normal physiologic condition of the liver, these cells exhibit a quiescent phenotype and are characterized by storing exceptionally high amounts of retinoids (vitamin A). On the other hand, injuries to the liver trigger the transdifferentiation of HSC, leading these cells to the activated phenotype that is characterized by the lost of vitamin A storing capacity and by the development of a myofibroblast-like morphology (Friedman and Hao 2017). This scenario represents a dramatic phenotypic change by which myofibroblasts-like HSC, are characterized by the upregulated expression of alpha-smooth muscle actin (α-SMA), collagens I/III and GFAP, by the imbalance between metalloproteinase

inhibitors (TIMPs) and matrix metalloproteinases (MMPs) and by the increased cell proliferation and cytokine releasing (Lee, Seo et al. 2004, Ramm, Shepherd et al. 2009, Kordes, Sawitza et al. 2014).

Resveratrol (RSV; 3,5,4′-trihidroxiestilbeno is a phytochemical that exhibits many beneficial properties and is found in grapes, wine, peanuts, tea, and berries (Burns, Yokota et al. 2002). RSV integrates a group of phytoalexins present in bark and is produced by plants under stress conditions (Orallo 2006). RSV exists in two forms: *cis* and *trans*. It is noteworthy that the *trans* isomer is the most stable steric form. Notably, the *trans* form is dominant in terms of prevalence and different biological activities are attributed to it, for example: the cell cycle arrest, the cell differentiation and apoptosis, and the antiproliferative effect in cancer cells (Orallo 2006, Anisimova, Kiselevsky et al. 2011, Nawaz, Zhou et al. 2017). Moreover, RSV participates on regulating lipid metabolism, protects cardiovascular tissue, inhibits platelet aggregation, and triggers anti-inflammatory and antioxidant effects (Rauf, Imran et al. 2017). Studies have shown that RSV can inhibit the initiation and the progression of tumours (liver cancer and breast cancer) (Tang, Su et al. 2008, Lee, Shin et al. 2010). However, the molecular mechanism for the therapeutic effect of RSV in HSC is unclear.

In recent years, the use of computational resources in the area of scientific research is growing. Studies provided a very useful framework for mapping and studying specific genetic variants that contribute for biologic events, in particular, the molecular mechanism of cellular events and diseases (van Kampen and Moerland 2016, Guo, Xiao et al. 2017). Nowadays, there is a wide range of choice for databases that are available *online* and can be used for bioinformatics analysis. This methodological approach makes possible to capture and to predict regulation by RNA or by RNA–protein interactions and to identify important trends and patterns of specific

gene and protein targets on which cells depend, thus allowing the identification of drug targets and actions (Ram, Mendelsohn et al. 2012, Shangguan, Tan et al. 2015, Chen, Huang et al. 2017, Guo, Xiao et al. 2017). Especially, the drug database shows a comprehensive information about drugs that makes possible to predict *in silico* the action of drugs and chemicals in biological models. Therefore, this analysis provides us a good opportunity to analyse new targets for drugs development and a better cognition of the molecular mechanism or pharmalogical action to find new application of existence drugs. Also, this technique allows the identification of pathways that are affected by studied drugs.

In this study, we investigate the targets of RSV, using the integrated bioinformatics tools as an effective approach, in the Drugbank database. Then, it was analysed the Protein-Protein interaction of RSV targets. Subsequently, it was performed the analysis of RSV-associated pathways. Finally, it was analysed the expression of this genes in the HSC microarray.

2 Material and Methods

2.1 Recognition of direct protein targets (DPTs) for Resveratrol

The strategic design used in this study is showed in the figure 1. For the recognizing the DTPs of RSV, it was used the DrugBank database (https://go.drugbank.com/). This is a *on line* freely available web resource database that combines drug data with information at clinical level, focusing in drug effects and interactions at molecular level, thus allowing to establish what proteins a drug interacts

(Wishart, Knox et al. 2008). This approach also offers additional information such as the ways in which drugs work and their indication (Knox, Law et al. 2011). The present version 5.1.7 was released at 07-02-2020 and contains 13,791 drugs databases, including 1,417 approved biologics data (proteins, peptides and allergenics), 2,653 small molecule drugs, 6,451. Additionally, 5,236 non-redundant protein (i.e. drug target/enzyme/transporter/carrier) sequences are linked to these drug entries (Wishart, Feunang et al. 2018). To search the DTPs, it was used the term “resveratrol” and it was selected the targets.

2.2 Protein-Protein interaction (PPI) network and signalling pathways of Resveratrol DTPs analysis

For building the PPI network, it was used the STRING database (https://string-db.org/). This database includes 5,090 organisms, 24.6 millions of protein-related data and >2000 millions of interactions (Szklarczyk, Morris et al. 2017). Also, this database is a very friendly on-line database and offers many options of analysis. The PPI is widely used for underlying physiological mechanism for predicting interactions and actions of proteins in an organism. The identification and characterization of PPI in different experimental situations is necessary to better understand their physiology and to determine their efficacy; among them, the direct (physical) protein interactions, the indirect (functional) protein interactions, and both the specific and biological significate in the organism or biology system (Szklarczyk, Franceschini et al. 2011). Data from String database was downloaded and analysed using the CYTOSCAPE® software, which is an open source project for building biomolecular integrating network program (Otasek, Morris et al. 2019). The PPI network for DTPs of RSV targets was constructed

by STRING APP of CYTOSCAPE®. The pathway analysis of RSV targets was used Reactome database with ReactomeFIVIz app. The used significance was P<0.05.

2.3 Identification of potential therapeutic targets genes for Resveratrol in HSC

After the identification of DTPs for RSV, we explore the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed). The PubMed database has a search engine that provides access for the biomedical literature, which contains bibliographic information of more than 27 million articles from more than 7,000 journals, including full text for approximately 4 million of scientific articles (Sayers, Beck et al. 2020). In this study we utilized the bibliographic research to find the DTPs for RSV, since that there is no database involving HSC.

3. Results

3.1 Identification of DTPs for Resveratrol

RSV was described to have anti-oxidative properties through scavenging reactive oxygen species (ROS). Also, RSV is widely recognized for its anti-aging as well as for its anti-cancer effects, anti-inflammatory properties, and anti-neurodegeneration properties. In DrugBank database, we found 26 primary direct DTPs for RSV: NQO2, CSNK2A1, PTGS1, PTGS2, ALOX15, ALOX5, AHR, PI4K2B, ITGA5, ITGB3, APP, SNCA, SIRT1, ESR1, MTNR1A, MTNR1B, CLEC14A, NR1I2, NR1I3, SLC2A1, CBR1, PPARA, PPARG, AKT1, KHSRP and YARS (Table 1).

Table 1 Identification of directs targets od Resveratrol using DRUGBANK.

DB_ID	*Name*	*Target_Symbol*	*Uniprot ID*	*Name*
DB02709	Resveratrol	NQO2	P16083	Ribosyldihydronicotinamide dehydrogenase
		CSNK2A1	P68400	Casein kinase II subunit alpha
		PTGS1	P23219	Prostaglandin G/H synthase 1
		PTGS2	P35354	Prostaglandin G/H synthase 2
		ALOX15	P16050	Arachidonate 15-lipoxygenase
		ALOX5	P09917	Arachidonate 5-lipoxygenase
		AHR	P35869	Aryl hydrocarbon receptor
		PI4K2B	Q8TCG2	Phosphatidylinositol 4-kinase type 2-beta
		ITGA5	P08648	Integrin alpha-5
		ITGB3	P05106	Integrin beta-3
		APP	P05067	Amyloid beta A4 protein
		SNCA	P37840	Alpha-synuclein
		SIRT1	Q96EB6	NAD-dependent protein deacetylase sirtuin-1
		ESR1	P03372	Estrogen receptor
		MTNR1A	P48039	Melatonin receptor type 1A
		MTNR1B	P49286	Melatonin receptor type 1B
		CLEC14A	Q86T13	C-type lectin domain family 14 member A
		NR1I2	O75469	Nuclear receptor subfamily 1 group I member 2
		NR1I3	Q14994	Nuclear receptor subfamily 1 group I member 3
		SLC2A1	P11166	Solute carrier family 2, facilitated glucose transporter member 1
		CBR1	P16152	Carbonyl reductase [NADPH] 1
		PPARA	Q07869	Peroxisome proliferator-activated receptor alpha
		PPARG	P37231	Peroxisome proliferator-activated receptor gamma
		AKT1	P31749	RAC-alpha serine/threonine-protein kinase
		KHSRP	Q92945	Far upstream element-binding protein 2
		YARS	P54577	Tyrosine--tRNA ligase, cytoplasmic

3.2 Caracterization the link between RSV DTPs.

The PPI and signalling pathways of 26 DTPs for RSV were generated with APP String in the CYTOSCAPE® software (Figure 2). The network analysis showed 26 nodes and 56 edges; and this suggested a great relationship among RSV and DTPs.

The FoamTree graph was constructed to have a better visualization of pathways (Figure 3). The top 5 Pathway analyses of DTPs was Nuclear Receptor transcription pathway, Generic Transcription Pathway, RNA Polymerase II Transcription, Interleukin-4 and Interleukin-13 signalling and Gene expression (Transcription) (Table 2).

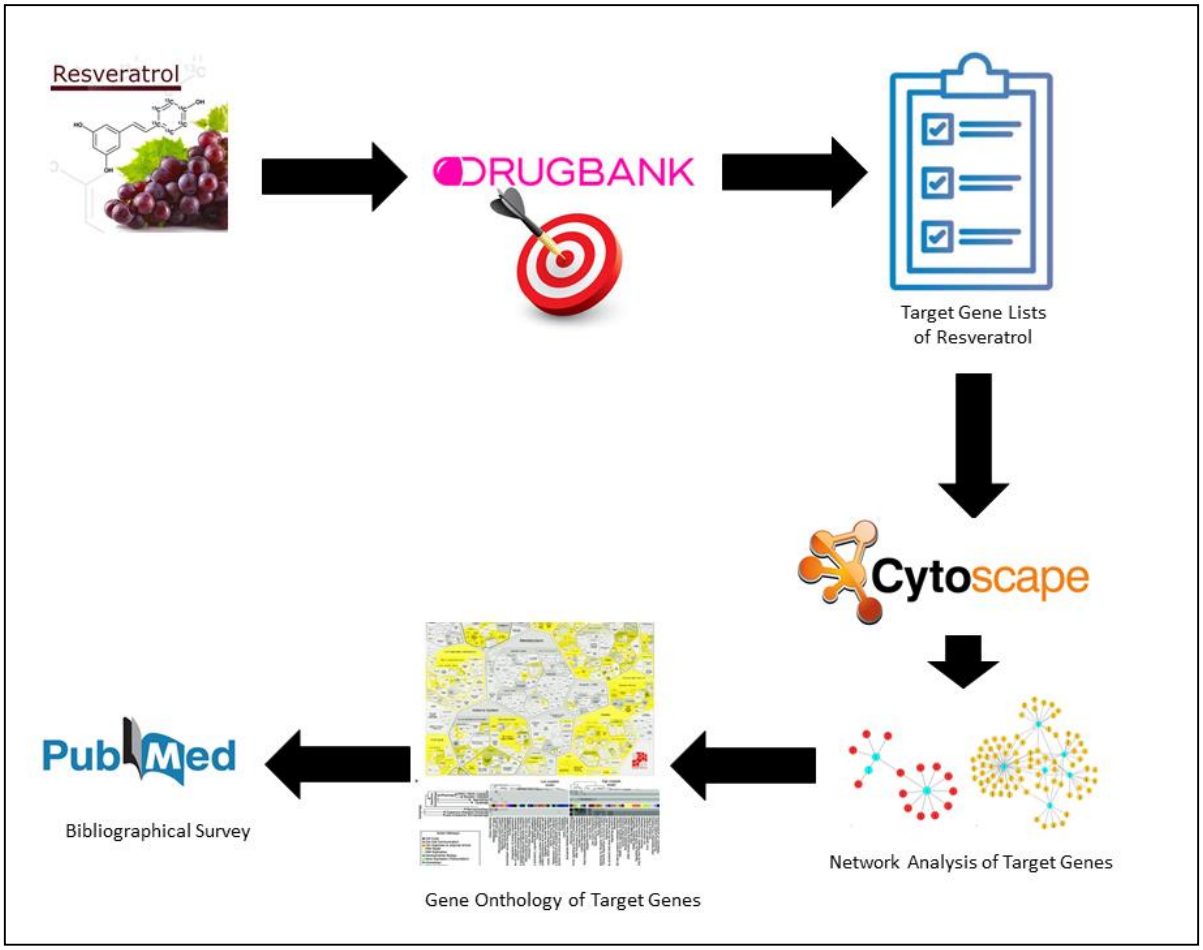

Figure 1: Workflow of methodology of this study.

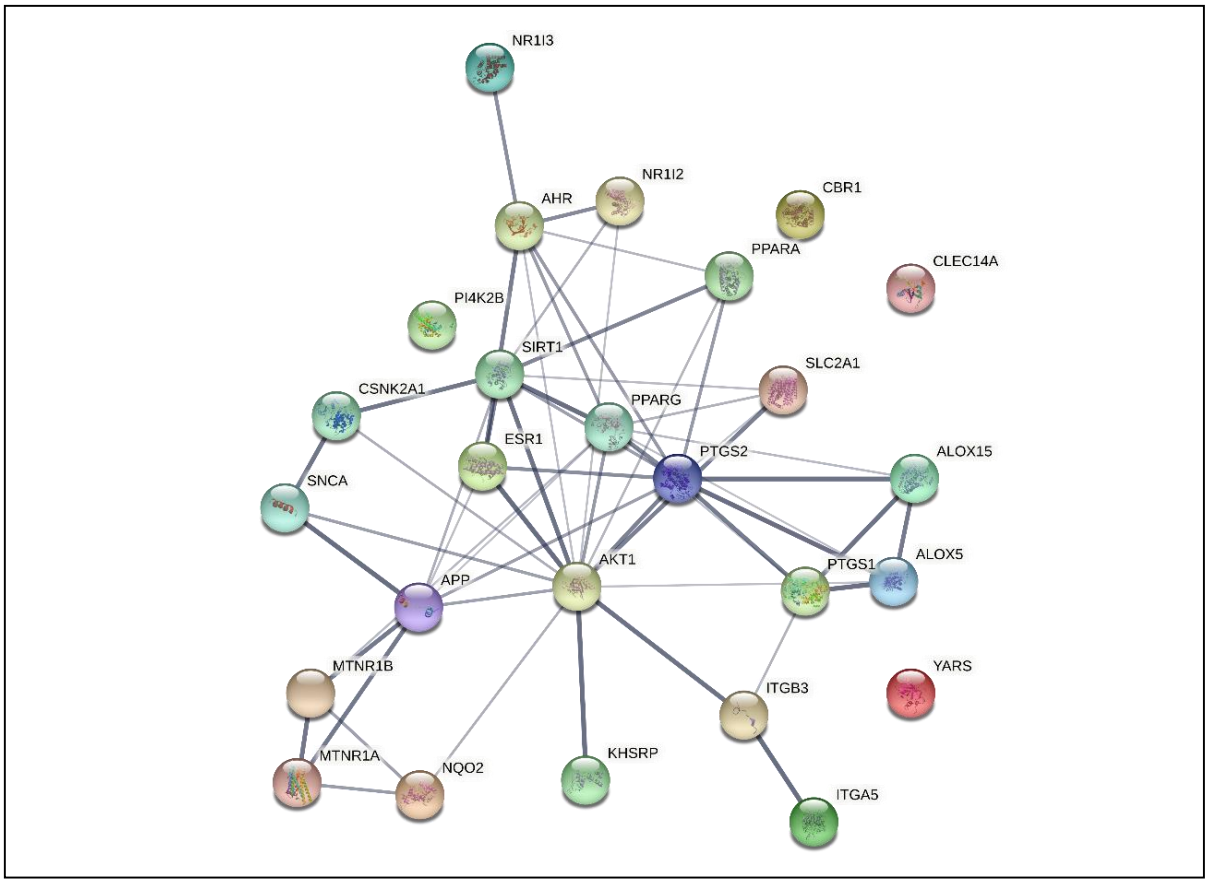

Figure 2: The PPI network of resveratrol target-genes (NQO2, CSNK2A1, PTGS1, PTGS2, ALOX15, ALOX5, AHR, PI4K2B, ITGA5, ITGB3, APP, SNCA, SIRT1, ESR1, MTNR1A, MTNR1B, CLEC14A, NR1I2, NR1I3, SLC2A1, CBR1, PPARA, PPARG, AKT1, KHSRP and YARS)[cutoff = 9).

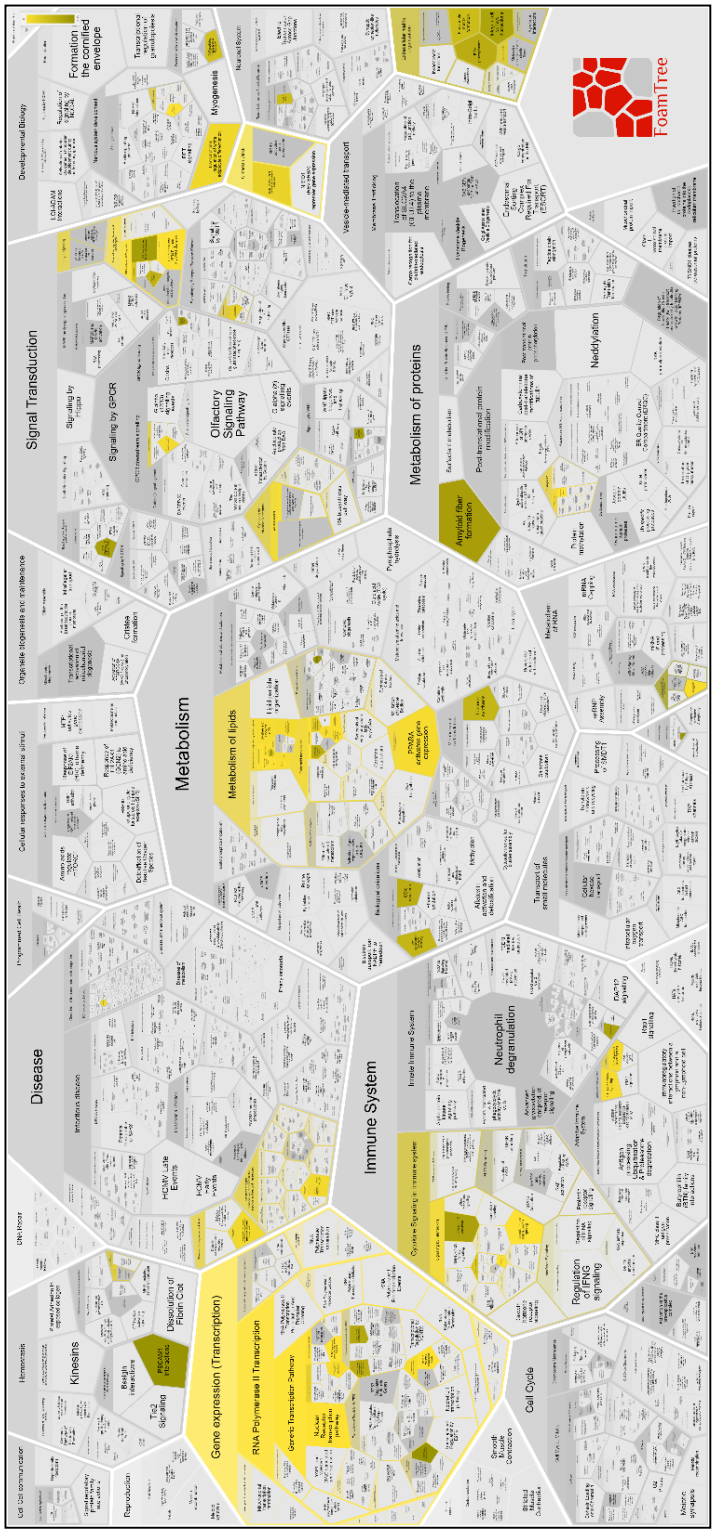

Figure 3: Visualization of 26 DTPs of resveratrol target genes (NQO2, CSNK2A1, PTGS1, PTGS2, ALOX15, ALOX5, AHR, PI4K2B, ITGA5, ITGB3, APP, SNCA, SIRT1, ESR1, MTNR1A, MTNR1B, CLEC14A, NR1I2, NR1I3, SLC2A1, CBR1, PPARA, PPARG, AKT1, KHSRP and YARS) with Foamtree graph of Reactome database. The most significates pathways are represented in the yellow tone; how significant the route is the darker the more significant.

Table 2: Top 5 Pathway analysis of DTPs of resveratrol.

Pathway name	***Found***	***p-value***	***FDR****
Nuclear Receptor transcription pathway	12	$3.33e^{-16}$	$1.23e^{13}$
Generic Transcription Pathway	20	1.83e-07	3.39e-05
RNA Polymerase II Transcription	20	7.37e-07	9.07e-05
Interleukin-4 and Interleukin-13	8	1.06e-06	9.74e-05
Gene expression (Transcription)	20	3.01e-06	2.20e-04

4. Discussion

Liver fibrosis is a reversible wound-healing process and has an important role on maintaining organ integrity (Bataller and Brenner 2005). However, chronic liver fibrosis may eventually culminate in liver failure, which has as only alternative treatment the liver transplantation (Khomich, Ivanov et al. 2019). Liver fibrosis is generally triggered by chronic hepatic injury caused by several factors, mainly viral infection (hepatitis B or C), schistosomiasis, and exacerbated consumption of alcohol (Zoubek, Trautwein et al. 2017).

The activated HSC (aHSC) are the major source of collagen I in the liver and secrete ECM proteins, inhibitors of metalloproteinases, and matrix metalloproteinases (MMPs) that elicit liver architecture remodelling. It is important to note that aHSC are responsible for 80% of total fibrillar collagen I in the fibrotic liver; thus, the aHSC

depletion is critical for the resolution of liver fibrosis. Furthermore, a large number of drugs have been explored for the management of these cells.

RSV has been implicated as the most important polyphenol responsible for the beneficial effects in several diseases through exerting anti-oxidative, anti-neoplastic and anti-inflammatory effects (Khomich, Ivanov et al. 2019). Further, RSV has been shown to have anti-fibrogenic activity by preventing TGF-β (Olson, Naugle et al. 2005). Other study showed that RSV significantly reduced mortality, transaminase concentrations in blood serum and liver lesions in mice, while it improved health and survival in obese mice (Baur, Pearson et al. 2006, Bujanda, Garcia-Barcina et al. 2006). These facts suggested RSV to be a benefic candidate for treating chronic liver diseases. However, its effects on HSC still remain unclear.

In this work, we present a survey from public databases using the current bibliography. In this way, we seek for a better understanding of RSV effects in HSC; its role in the phenotypic modulation of this cell. Thus, 26 RSV target genes and the main pathways for this phytoalexin were identified. This bibliographic survey demonstrated that 19 genes targets of RSV were not found in the scientific portal PUBMED when taking into account its role in the phenotypic modulation of HSC. On the other hand, 7 DTPs have already been associated to studies found in the PUBMED database.

The ALOX5 gene acts in the major products of arachidonic acid metabolism and participates in inflammatory responses. It was already reported that knockout of ALOX5 can improve the inflammatory process during fibrotic responses in Apoe-/- mice (Martinez-Clemente, Ferre et al. 2010). Similar results on decreased expression of ALOX5 was shown in response to CCl4-induced liver fibrosis (Titos, Ferre et al. 2010). Chemical inhibitors of ALOX5 have been shown to reduce CCl4-induced liver

injury through decreasing the inflammatory infiltration of hepatic parenchyma in several distinct models of NASH and NAFLD (Martinez-Clemente, Ferre et al. 2010). The isoform AKT1 plays several roles in inflammation, cell proliferation, migration, and fibrogenesis during LPS treatment (Reyes-Gordillo, Shah et al. 2019). However, there are no studies that associate the performance of ALOX5 and AKT1 specifically, specifically wich the phenotypic modulation of HSC.

The role of AHR in liver fibrosis is controversial: both the presence and the absence of this protein lead to liver fibrosis (He, Hu et al. 2013). This receptor is highly expressed in the liver and regulates the xenobiotic metabolism, the inflammation, and the cell proliferation and death (Beischlag, Luis Morales et al. 2008). A study showed that AHR presented an anti-fibrogenic transcriptional factor in human and mouse HSC by preventing HSC activation and expression of genes required for liver fibrogenesis (Yan, Tung et al. 2019). ITGAB3 becomes expressed during HSC activation *in vitro*, and is responsible for promoting cell proliferation and survival. Its degradation is associated to ligands related to the resolution of liver fibrosis and may contribute to the observed apoptotic depletion of HSC (Zhou, Murphy et al. 2004).

The SIRT1, PPARγ and PPARα have a well-documented participation in the phenotypic modulation fn HSC. The PPARγ is primarily expressed in adipose tissue and promotes the transcription of proteins that induce lipid storage and adipogenic differentiation (Desvergne 2008). HSC cells show a significant decrease in PPARγ expression during the transition to the activated state. Conversely, the induction of increased PPARγ expression is sufficient to reestablish several markers of the quiescent phenotype. This fact suggests an important role for PPARγ in maintaining the lipocytic phenotype (Guimaraes, Franceschi et al. 2007, Bitencourt, de Mesquita et al. 2012). It is interest to note that SIRT1 interacts with PPARγ through the nuclear

coreceptor N-CoR and it is related to the regulation of lipid metabolism and the decrease of adipogenesis (Picard, Kurtev et al. 2004). SIRT1 is also related to the modification of PGC-1A, a PPARγ coactivator, to maintain hepatic glycolytic homeostasis. In a previous study, we demonstrated that the lipogenesis and the consequent formation of lipid droplets in GRX cells only occur when the PPARγ/SIRT1 ratio is greater than 1 at the gene and protein levels (de Souza, Martins et al. 2015). PPARα expression has key roles in regulating the fatty-acid transport and participates in the peroxisomal and mitochondrial β-oxidation in the liver (Xu, Xiao et al. 2002). Notably, this situation is an important process for activation of HSC, when these cells lose theirs lipid droplets (Friedman and Hao 2017). Also, it has already been demonstrated that PPARα knockout in mice increases the susceptibility of liver steatosis, inflammation, and hepatocellular carcinoma (Abdelmegeed, Yoo et al. 2011, Lalloyer, Wouters et al. 2011).

Interestingly to note that most of DTPs for RSV integrates many pathways, but there are only top 5 in our pathway analyses. Notably, RSV can act in several regulation pathways for Cell Signaling such as the TGFβ1/SMAD Pathway, WNT Pathway, SHH/GLI Pathway, NOTCH Pathway, STAT Signaling, AKT, and MAPK and ATM/p53 Pathway (Farooqi, Khalid et al. 2018, Malaguarnera 2019, Pannu and Bhatnagar 2019). Therefore, these findings suggest the RSV property on increasing cell signalling and promoting important molecular changes for HSC. In summary, this study demonstrated that Bioinformatics analysis provide a simple and flexible form to investigate hypothesis of biological action of drugs. Nevertheless, we showed that DTPs of RSV still needs research for clarification of this genes on influencing the HSC phenotype.

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CAPÍTULO 3

***Manuscrito de Bioinformática atualmente em validação experimental.**

Bioinformatics analysis of high significant gene expression involved in the hepatic stellate cells

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Abbreviations

DEG	Differential expression gene
PPI	Protein-protein interaction
GO	Gene onthology
HSC	Hepatic stellate cells
qHSC	Quiescent hepatic stellate cells
aHSC	Activated hepatic stellate cells
ECM	Extracellular cell matrix
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
α-SMA	α-smooth muscle actin
GEO	Gene Expression Omnibus
BP	Biological process
MF	Molecular function
CC	Cellular component
LIMMA	Linear Models for Microarray Data
PANTHER	Protein Analysis Through Evolutionary Relationships
GREM1	Gremlin 1
RGS4	Regulator of G-protein signaling 4
LOX	Lysyl oxidase
PTX3	Pentraxin 3

POSTN	Periostin, osteoblast specific factor
SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
ITGA11	Integrin, alpha 11
INHBA	Inhibin, beta A
ORM1	Orosomucoid 1
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
ALB	Albumin
APOA2	Apolipoprotein A-II
FGA	Fibrinogen alpha chain
HP	Haptoglobin
FGB	Fibrinogen beta chain
APOC1	Apolipoprotein C-I
ORM2	Orosomucoid 2
PECAM1	Platelet/endothelial cell adhesion molecule

ABSTRACT

Hepatic Stellate Cells (HSC) activation is a crucial event during liver fibrosis development. Here, we investigated high significates gene expression profile of HSC using bioinformatics tools for obtaining a deeper understanding on the molecular mechanism and pathways involved with cell activation. GSE68000 microarray data were downloaded from the Gene Expression Omnibus database. Samples from quiescent and activated HSC were evaluated. Differential expression genes (DEG) was calculated with LIMMA package for R and included 411 genes, being 155 upregulated and 256 downregulated. These genes were submitted to Gene ontology annotation and Reactome pathways analysis. Protein-Protein Interaction was established with Cytoscape software and module analysis was performed with the MCODE plugin. In summary, the major upregulated genes were mainly described in literature to be involved with HSC activation or liver fibrosis development. Therefore, there is a need to study the roles of the major downregulated genes at HSC physiology. In the frame of bioinformatics data mining approaches, this study showed that high expressed genes in HSC had important components of phenotype transdifferentiation of HSC that serve better understand the processes associated with liver fibrosis.

Keywords: Hepatic fibrosis, Hepatic stellate cells, Differential gene expression, Bioinformatics, biological pathways, network analysis.

Highlights

Few genes differentially expressed are described as acting in the HSC phenotype modulation.

Some of high expressed genes are described as markers of liver fibrosis, but not in phenotype modulation.

Some of high expressed genes in HSC act in autocrine signaling.

It is not yet clear the role of most differentially expressed genes in their role in HSC transdifferentiation.

1. INTRODUCTION

Liver fibrosis is a complex process that characterizes the response to persistent liver injuries, which can lead to accumulation of extracellular matrix (ECM) and to chronic inflammatory process [1]. The cellular process of fibrogenesis can be triggered in response to an acute liver damage to reestablish hepatic structural integrity. Sustained noxious stimuli in chronic injury may lead to an alteration of liver parenchyma with progressive deterioration of liver function [2]. Depending on its persistence, fibrogenesis plays a key role in the development of hepatic cirrhosis and hepatocellular carcinoma. Various etiologies can lead to the initialization of this condition; among them, genetic diseases, viral infections (i.e., hepatitis B and C), autoimmune conditions, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcohol abuse, and toxic damage [3,4].

Hepatic stellate cells (HSC) account for 5%–8% of the cells in the liver and are located in the perisinusoidal space (space of Disse) [5]. These cells are known for exerting a pivotal action in the development of liver fibrogenesis and present two distinct morphological phenotypes [6]. Quiescent hepatic stellate cells (qHSC) are predominant in the normal liver and contain a considerable number of lipid droplets in the cytoplasm, which store 70% of total retinoid found in the body [7]. On the other hand, activated hepatic stellate cells (aHSC) are predominant in the injured liver. During the activation process, qHSC cells lose their lipid droplets, differentiating to myofibroblast-like cells that are responsible for producing an excessive extracellular matrix enriched with collagen type I. Comparing to qHSC, aHSC present an increased rate of cell proliferation and migration and an increased expression of α-smooth muscle actin (α-SMA), which sustains its myofibroblast-like morphology [5,8,9].

A variety of biological factors is involved in the pathogenesis of liver fibrosis. Thus, a comprehensive understanding on the HSC phenotype modulation that lead to fibrogenesis is a challenge due to the process complexity, which especially involves the intricate regulation of gene expression [10]. In this way, microarray analysis has been widely used in the screening of differentially expressed genes (DEG) as an approach to investigate the multiple signaling pathways that may contribute to liver fibrosis development [11–13]. Microarray analysis enables the investigation of the biological mechanism of HSC activation that have not been previously done [3], allowing for a better understanding on the gene expression and biological pathways that act in different contexts [14]. Thus the in silico study on how HSC behave in normal liver or how these cells respond to liver injuries by evaluating the global gene expression can facilitate the discovery of new potential biological targets for liver fibrosis treatment [11–13].

In the present study, we aimed to identify variations in the genetic expression through analyzing the highest statistical significance ($P<0.001$ and $LogFC>4$) and the pathways that act in the transdifferentiation of hepatic stellate cells. To reach these objectives, we use the GSE68000 microarray data for analyzing and comparing the differential expression genes (DEG) of uncultured qHSC and in vitro cultured aHSC. Thereafter, we analyzed the related gene ontology (GO) enrichment terms and pathways, and further constructed protein–protein interactions (PPI).

2. MATERIAL AND METHODS

2.1 Microarray data

The GSE68000 microarray data were downloaded from Gene Expression Omnibus (GEO) database, which was based in the platform GPL13667 ([HG-U219] Affymetrix Human Genome U219 Array) and deposited by El Taghdouini et al. [15]. Three samples of human qHSC and aHSC primary cultures were selected.

2.2 Analysis of Differential Expression Genes (DEG)

Raw expression data were analyzed using the statistical software R (version 3.5.3, https://cran.r-project.org/) and the necessary packages from Bioconductor (https://www.bioconductor.org/). Samples were downloaded with GEOquery in R/Bioconductor package [16]. The background correction and “rma” normalization of all selected data was performed with the “affy” R package [17]. Then, qHSC and aHSC samples were submitted to significance analyses for DEG, using Linear Models for Microarray Data (LIMMA package) [18]. *P*-value was adjusted according to Benjamini’s method [19] and calculated as Log2-Fold change. In this study, it was considered significant the adjusted $P<0.001$ and the LogFC>4, to obtain the genes with the highest statistical significance. Figure 1 resumes the methodology steps used in this work.

----------Insert Figure 1----------

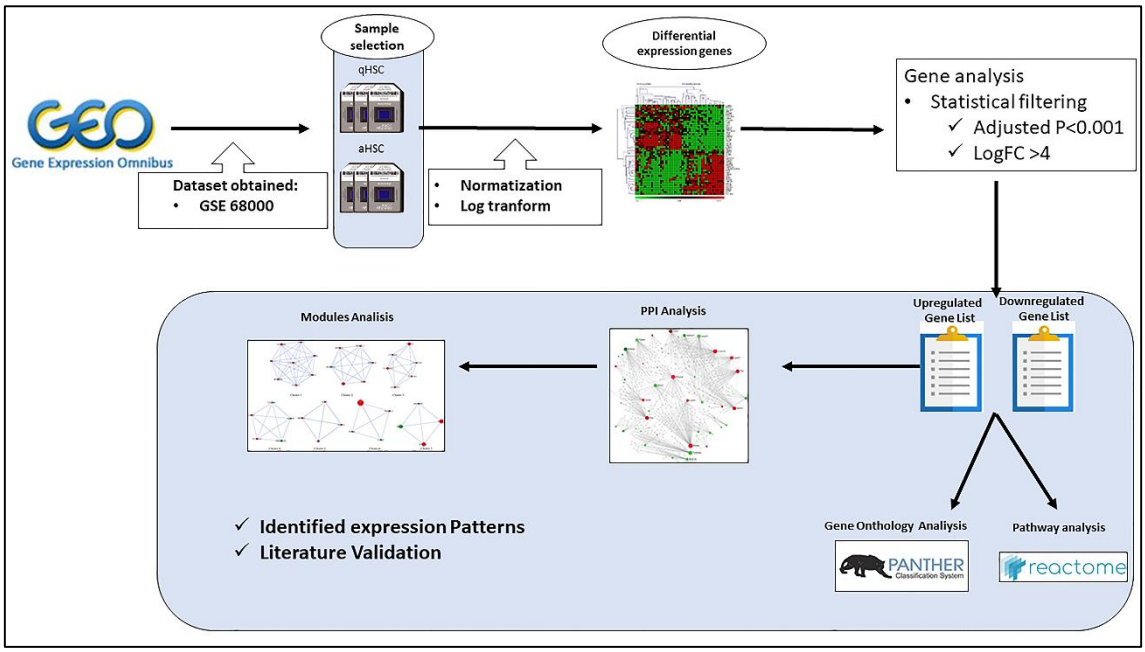

Figure 1. Workflow: Summary of the methodological steps used in this work.

1.1 Analysis of Gene Ontology (GO) enrichment terms and Reactome pathway

To classify the selected DEG, the online database Protein Analysis Through Evolutionary Relationships (PANTHER) [20] was used and gene ontology (GO) was conducted for gene annotation in biological process (BP), molecular function (MF), and cellular component (CC). Further, the Reactome Pathway (http://www.pantherdb.org/) was used for analyzing pathways on which these genes are involved. Results for GO and Reactome pathways in the obtained DEG were considered significant when $P<0.05$, considering Bonferroni correction for multiple testing as <0.05.

1.2 Construction of Protein-protein interaction (PPI) Network

PPI network of selected upregulated and downregulated genes from DEG analysis was constructed using the software Cytoscape version 3.7.1 (https://cytoscape.org/index.html) by String App [21]. Interactions whose integrated scores were >7 in STRING database version 11.0 (https://string-db.org) were selected. Then, the MCODE plugin was used to analyze the specific bio-functional modules and sub-modules present on PPI.

2. RESULTS

2.1 Screening of Differentially Expressed Genes (DEG)

DEG analysis was utilized to compare gene expression levels in three samples of qHSC and three samples of aHSC. The GSE68000 dataset was standardized and is showed in Figure 2. We identified a total of 411 DEG, being 155 upregulated genes and 256 downregulated genes that were significantly expressed (supplementary table I). The top 10 most altered expression for upregulated and downregulated genes are listed in Table I.

----------Insert Figure 2----------

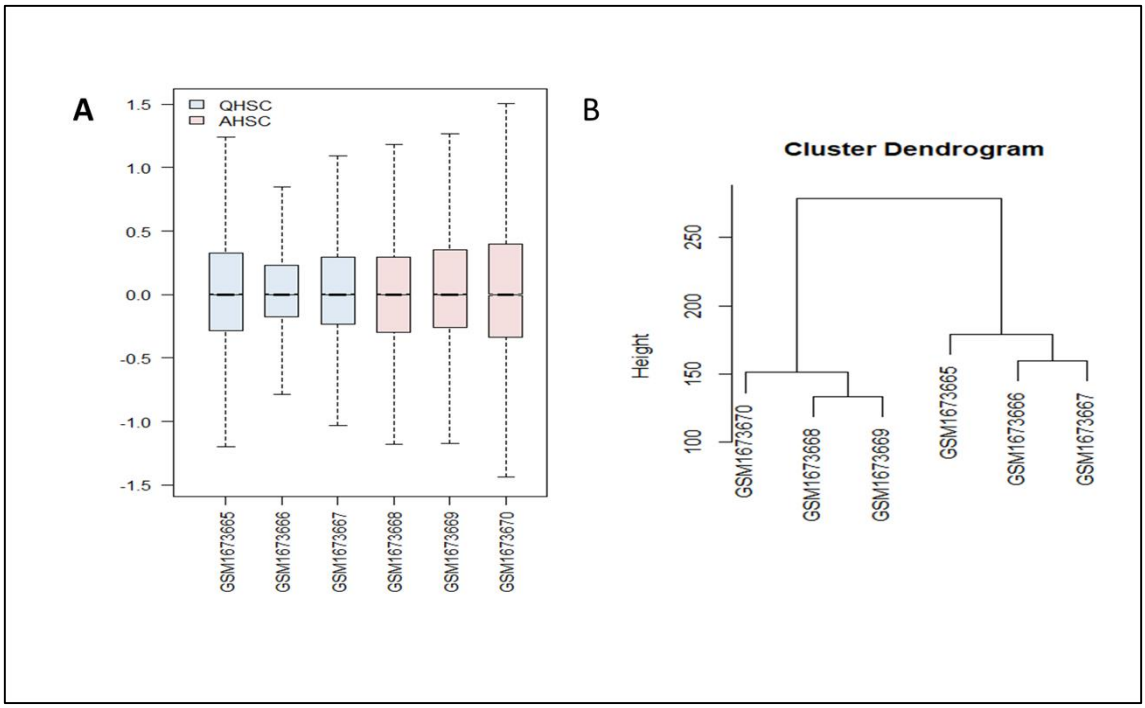

Figure 2. (A) Box plot for the sample data. The name of each sample is arranged at the x axis and the variation of gene expression is arranged at the y axis. Blue bars represent quiescent hepatic stellate cells (qHSC) and pink bars represent activated hepatic stellate cells (aHSC). (B) Cluster dendrogram analysis of the samples.

----------Insert Table 1----------

Table I. DEG for the top 10 upregulated and downregulated genes from the GSE68000 microarray data.

	Gene symbol	GB LIST	LogFC	Adj.P.Val
Upregulated	***GREM1***	NM_013372	**7,338789**	**6,18E-05**
	RGS4	NM_001102445 NM_001113380 NM_001113381 NM_005613	**7,019502**	**0,000144**
	LOX	NM_001178102 NM_002317	**6,748825**	**0,00043**
	KIAA1199	NM_018689	**6,476977**	**0,001895**
	PTX3	NM_002852	**6,349377**	**0,001536**
	POSTN	NM_001135934, NM_001135935 NM_001135936 NM_006475	**6,294243**	**0,000926**
	SEMA3C	NM_006379	**6,292062**	**0,000217**
	UCHL1	NM_004181	**6,205153**	**0,001077**
	ITGA11	NM_001004439	**6,132888**	**0,000323**
	INHBA	NM_002192	**6,077769**	**0,000896**
Downregulated	***ORM1***	NM_000607 NM_000608	**-9,59985**	**0,00028**
	SERPINA1	NM_000295 NM_001002235 NM_001002236 NM_001127700 NM_001127701 NM_001127702 NM_001127703 NM_001127704 NM_001127705 NM_001127706 NM_001127707	**-9,5153**	**0,000124**
	ALB	NM_000477	**-9,48161**	**9,56E-06**
	APOA2	NM_001643	**-9,31185**	**2,38E-05**
	FGA	NM_000508 NM_021871	**-9,20685**	**0,000158**
	HP	NM_001126102 NM_005143	**-8,61953**	**0,000322**
	FGB	NM_001184741 NM_005141	**-8,52279**	**0,00033**
	APOC1	NM_001645	**-8,0405**	**0,00011**
	ORM2	NM_000608	**-8,0319**	**0,00133**
	PECAM1	NM_000442	**-7,9010**	**2,99E-05**

1.1 Gene Ontology (GO) enrichment terms for Differentially Expressed Genes (DEG)

GO enrichment terms may directly reflect the distribution of gene expression, thus impacting in the Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC) [22]. Upregulated and downregulated genes from the obtained DEG were analyzed in PANTHER database (version 14.0) and ten significantly GO enrichment terms were organized by number of genes.

For upregulated genes (Table II), the GO enriched terms mainly were: Binding (P= 1.52E-02), Cellular process (P= 3.39E-02), Protein binding (P= 4.61E-02), Multicellular organismal process (P= 9.88E-07), and Developmental process (P= 9.37E-10). For downregulated genes (Table III), the GO enriched terms mainly were: Biological regulation (P= 1.86E-02), Response to stimulus (P= 3.95E-09), Multicellular organismal process (P= 1.49E-03), Extracellular region (P= 2.73E-13), and Localization (P= 3.01E-06).

For the upregulated genes, the active pathways found at Reactome database were: Extracellular matrix organization (P= 5.47E-06), Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs) (P= 2.28E-02), Collagen formation (P= 3.70E-03), Integrin cell surface interactions (P= 4.94E-02), Assembly of collagen fibrils, and other multimeric structures (P= 1.06E-02). For downregulated genes, the active pathways found at Reactome database were: Immune System (P= 1.06E-02), Hemostasis (P= 1.06E-02), Innate Immune System (P= 1.06E-02), Neutrophil degranulation (P= 1.06E-02), and G alpha (i) signaling events (P= 3.04E-03) (Table IV).

----------Insert Table II----------

Table II. Top 10 GO enrichment terms for upregulated genes.

Category	Description/Term	Count	P-value
MF	**Binding (GO:0005488)**	**76**	**1.52E-02**
BP	**Cellular process (GO:0009987)**	**74**	**3.39E-02**
MF	**Protein binding (GO:0005515)**	**65**	**4.61E-02**
BP	**Multicellular organismal process (GO:0032501)**	**55**	**9.88E-07**
BP	**Developmental process (GO:0032502)**	**54**	**9.37E-10**
BP	**Multicellular organism development (GO:0007275)**	**49**	**1.36E-08**
BP	**System development (GO:0048731)**	**45**	**7.36E-08**
BP	**Cellular developmental process (GO:0048869)**	**41**	**1.78E-07**
CC	**Extracellular region (GO:0005576)**	**40**	**2.61E-05**
BP	**Cell differentiation (GO:0030154)**	**38**	**8.20E-06**

----------Insert Table III----------

Table III Top 10 GO enrichment terms for downregulated genes.

Category	Description/Term	Count	P-value
BP	**Biological regulation (GO:0065007)**	**106**	**1.86E-02**
BP	**Response to stimulus (GO:0050896)**	**96**	**3.95E-09**
BP	**Multicellular organismal process (GO:0032501)**	**74**	**1.49E-03**
CC	**Extracellular region (GO:0005576)**	**72**	**2.73E-13**
BP	**Localization (GO:0051179)**	**71**	**3.01E-06**
CC	**Cell periphery (GO:0071944)**	**68**	**5.35E-05**
CC	**Plasma membrane (GO:0005886)**	**68**	**2.33E-05**
BP	**Positive regulation of biological process (GO:0048518)**	**66**	**1.19E-02**
CC	**Endomembrane system (GO:0012505)**	**65**	**1.72E-08**
CC	**Vesicle (GO:0031982)**	**65**	**6.67E-12**

----------Insert Table IV----------

Table IV. Top 5 active pathways for the upregulated and downregulated genes.

	Description	Reactome ID	Count	P-value
Upregulated	Extracellular matrix organization	R-HSA-1474244	12	5.47E-06
	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	R-HSA-381426	6	2.28E-02
	Collagen formation	R-HSA-1474290	6	3.70E-03
	Integrin cell surface interactions	R-HSA-216083	5	4.94E-02
	Assembly of collagen fibrils and other multimeric structures	R-HSA-2022090	5	1.06E-02
Downregulated	Immune System	R-HSA-168256	34	4.46E-02
	Hemostasis	R-HSA-109582	27	3.00E-05
	Innate Immune System	R-HSA-168249	24	1.46E-02
	Platelet degranulation	R-HSA-114608	14	2.38E-03
	G alpha (i) signalling events	R-HSA-418594	11	3.04E-03

3.3 Protein-Protein Interactions (PPI) network

The products of these 411 DEG were analyzed in Cytoscape Software, based on String database, in order to predict the interaction between genes. The PPI network revealed that downregulated genes presented 130 nodes and 466 edges (Figure 3A) while upregulated genes presented 79 Nodes and 21 edges (Figure 3B). Then, the PPI network for those downregulated and upregulated genes were analyzed using the MCODE plugin, and the modules were analyzed in accordance with the Reactome pathway database. It was found that downregulated genes showed four modules. Module A (Figure 4A) obtained a score of 20.96 and showed 26 nodes and 262 edges. These genes were related to Hemostasis pathways (P= 1.40E-18). Module B (Figure 4B) obtained a score of 5.00 and showed 5 nodes and 10 edges. These genes were

related to the pathway involved with signaling by receptor tyrosine kinases (P= 1.90E-03). Both module C and D (Figure 4C and Figure 4D) obtained a score of 3.00 and showed 3 nodes and 3 edges; however, these modules were not found in the Reactome pathway database. On the other hand, the upregulated genes showed one module. This module (Figure 3E) obtained a score of 5.60 and showed 6 nodes and 14 edges. These genes were related to the Extracellular matrix organization pathway (P= 1.22E-03).

----------Insert Figure 3----------

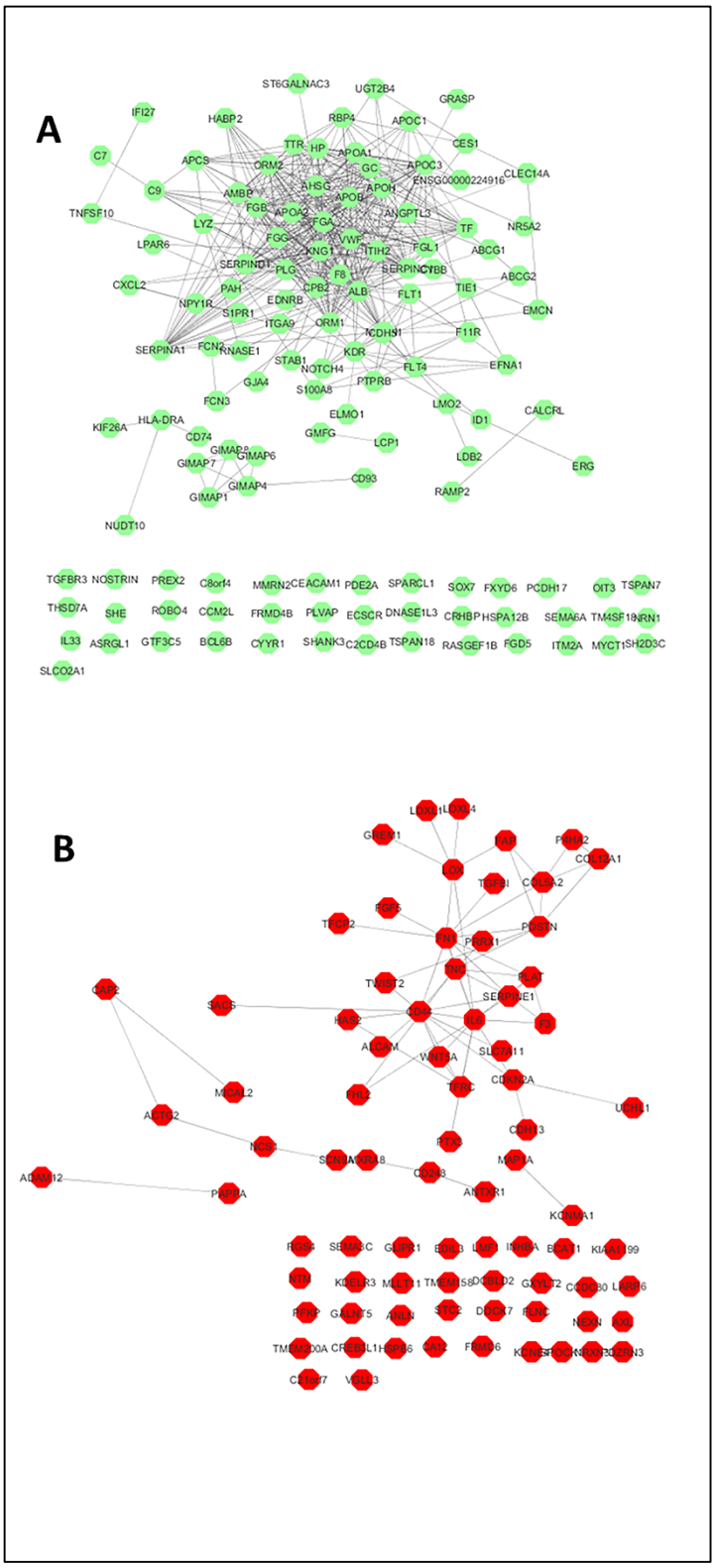

Figure 3. PPI network for DEG selected genes. Green nodes represent downregulated genes and red nodes indicate upregulated genes.

----------Insert Figure 4----------

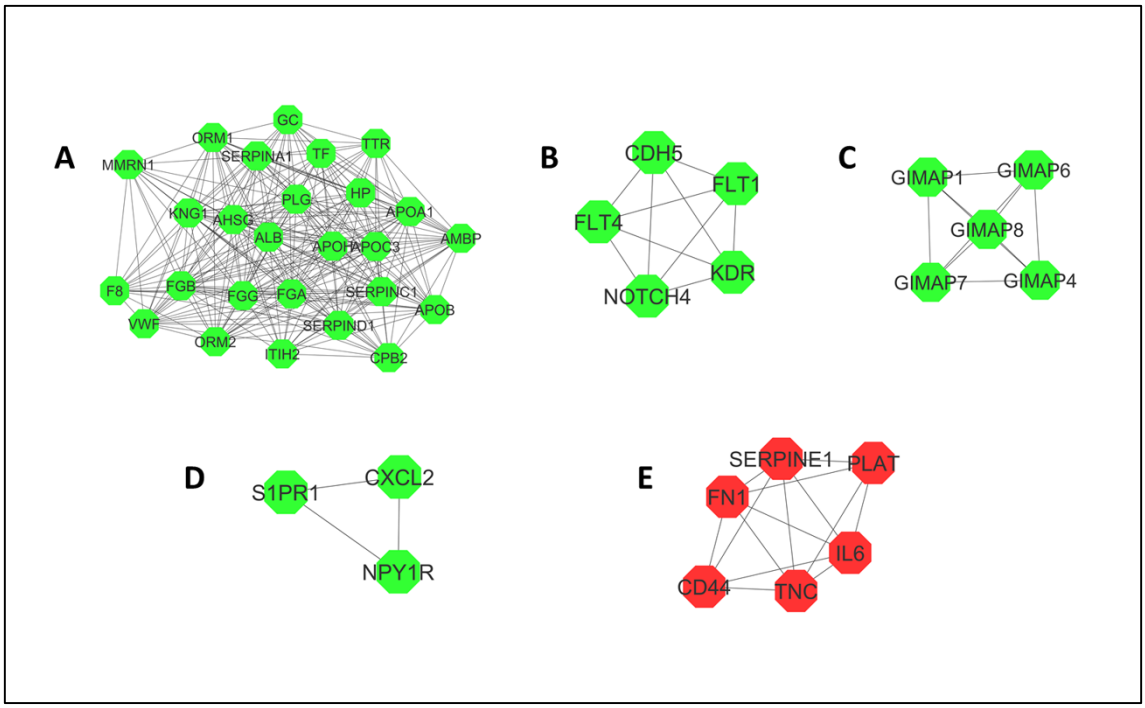

Figure 4. Module analysis for the selected genes. Green nodes represent downregulated genes and red nodes indicate upregulated genes.

3 DISCUSSION

In order to investigate the molecular mechanism associated to the most significant genes for the phenotypical modulation of HSC, we performed the present study using bioinformatics tools. Thus, a total of 411 differentially expressed genes (DEG) for qHSC and aHSC samples was identified by comparing their gene expression profiles; among them, we found 155 upregulated genes and 256 downregulated genes. In this study, it was analyzed the potential role of the 20 most significant genes (10 upregulated/10 downregulated) in our DEG analysis. Also, it was analyzed the DEG for gene ontology and further analysis was performed for constructing the Protein-protein interaction (PPI) network, intending to predict interactions between the product of DEG and relevant modules.

Notably, among the top 10 upregulated genes that were found with the used cutoff for this study (log fold<4/ adjusted P<0.001), most of them were previously described in the literature to be involved with HSC activation or liver fibrosis establishment. DEG analysis showed that Gremlin 1(GREM1) appeared with the highest genetic expression. Experimental evidences showed that GREM1 plays an important role on regulating organogenesis, body patterning, tissue differentiation, matrix-degrading enzymes expression, and bone morphogenetic proteins activity [23]. In HSC, this gene participates in the activation process through mediating the upregulation of TGF- β expression, which is an important marker of HSC activation [8,24]. Another study showed that induction of GREM1 expression may have a profibrotic effect, thus suggesting that its inhibition is beneficial on treating liver fibrosis [25], thus, emphasizing its importance in the HSC phenotypic transition process. Besides GREM1, our data demonstrate that the Regulator of G Protein Signaling 4 (RGS4) gene was also upregulated. RGS4 has been shown to play an important role in neural systems of patients that are affected by cirrhotic liver damage due to chronic alcohol abuse [26]. Our analysis indicates that RGS4 gene may also play an important role in hepatic fibrosis through possible modulating HSC function; however, this process is not yet clear.

The Lysyl Oxidase (LOX) gene family, which presented a considerably increased expression in this analysis, is associated to the enhancement of the connective tissue strength and integrity [7]. In this way, LOX family directly involved in the liver fibrosis development by participating in the stabilization of ECM structure in liver [27]. In agreement to this fact, a recent study showed that the knockdown of Lysyl Oxidase 1 (LOX1) can suppress both cell proliferation and fibrogenesis of TGF-β1-stimulated HSC through regulating the phosphorylation of Smad2/3 [28]. Mesarwi et al

demonstrated that LOX expression may serve as a biomarker of liver fibrosis in patients with severe obesity and nonalcoholic fatty liver disease [7]. Moreover, Periostin (POSTN) gene codifies a matricellular protein that mediates pleiotropic effects during inflammatory process, tissue injury, and wound healing [29]. Also, this matricellular protein activates signaling pathways during tissue repair and matrix remodeling via transmembrane integrins [30]. POSTN was recently emerged as a novel therapeutic target for hepatic fibrosis treatment since it was found to exert a potent profibrotic activity that is mediated by α integrin-like protein [31]. Also, the POSTN protein was able to significantly increase the expression of Collagen I, LOX, and LOXL, which may lead to the extracellular matrix accumulation during chronic liver diseases [32]. It was also suggested that both genes may play an important role in the fibrosis process. However, their expression in this study highlights their possible role in phenotypic modulation of HSC.

Another gene that is upregulated in this study was the Integrin Subunit Alpha 11 (ITGA11), a binding protein that is expressed during the differentiation of myofibroblasts and is responsible for the collagen organization [33]. This gene promotes an important link between the activation of contractile fibroblasts and the complex collagen network. ITGA11 is essential for modulating HSC transdifferentiation during liver fibrosis [34]. In the same way, knockdown of ITGA11 was able to inhibit activation, differentiation, migration, and contractility of HSC [35]. Another study demonstrated that ITGA11 expression was associated to TGF-β activation and that knockdown of ITGA11 was able to downregulate the expression of TGFβ superfamily genes in HSC [35]. Therefore, our analysis corroborates that upregulation of ITGA11 implicates in the HSC transdifferentiating.

The KIAA1199 gene is closely associated to the development of tumors with different malignancies including gastric cancer [36], prostate cancer [37], colorectal tumors [38], and hepatocarcinoma [39]. In present analysis, the KIAA1199 gene appeared to be upregulated in DEG analysis. The KIAA1199 knockdown was related to the decrease in the proliferation and migration of hepatocarcinoma cells, which can contribute to the regression of liver cancer and metastasis [40]. Due to its aforementioned roles, KIAA1199 upregulation in HSC could indicate an involvement of this gene in the activation process since an increased cell proliferation and migration is expected during liver fibrosis establishment [39]. However, the function of KIAA1199 in HSC physiology should be investigated to elucidate this issue.

HSC is susceptible to environmental changes and stimuli in the injured liver. Cells like hepatocytes, macrophages, biliary epithelial cells, and liver sinusoidal endothelial cells can release mediators to stimulate HSC activation through paracrine signaling [41]. In this way, Ubiquitin C- Terminal Hydrolase L1 (UCHL1) gene expression has been described to be induced in hepatocytes by HCV infection, leading to an increase of UCHL1 protein in the plasma of patients with chronic hepatitis C. Thus, it was suggested that UCHL1 protein can play a role in HSC activation and proliferation thorough paracrine signaling [42,43]. In a similar way, the Pentraxin (PTX3) gene has a differential expression in normal and injured liver. Release of the PTX3 protein by neutrophils was associated to the liver inflammation modulation responsible for triggering HSC activation in response to liver injury [44]. Both UCHL1 and PTX3 genes were found to be differentially expressed in qHSC and aHSC; this fact may suggest that HSC play an important role in the inflammatory process, thus contributing itself to HSC activation by an autocrine signaling way.

It should be noted that Inhibin Subunit Beta A (INHBA) protein can secreted by hepatocellular secretion of INHBA by exosomes can cause migration, proliferation and HSC activation [45]. Moreover, the INHIBA expression has function as a modulator that is involved with cirrhosis and fibrosis, by activation of the SMAD signaling pathway in response to liver tissue injury [46]. Notably that our results indicate that's considerable expression in HSC, suggesting that represent important role in response of liver damage and HSC activation.

It was notably that among the top 10 downregulated genes obtained by DEG analysis, only six genes are reported to be involved in phenotypic modulation or function of HSC. In this study, Serpin Family Member 1 (Serpin1) was found to be the most downregulated gene. Serpin1 is involved with apoptosis induction and regulation and play a key role in the balance between cell proliferation, cell death, inflammatory processes and during cancer development [47]. It is interesting to note that Serpin family was recently found to be an important signaling mediator during chronic liver diseases and liver carcinogenesis [48]. Novo et al [49] have demonstrated the evidence that hepatocyte releasing of Serpin1 during chronic liver disease can contribute to liver fibrogenesis by acting on HSC modulation. In this way, our result reinforces the role of serpin1 expression in phenotypic modulation of HSC.

Albumin (ALB) gene was also found to be downregulated in our DEG analysis. The ALB protein that is associated with hepatic cirrhosis [50]. Indeed, ALB expression has been widely used as a marker for mature hepatocytes and it was recently demonstrated the role of HSC on inducing hepatocyte progenitor cells differentiation into mature cells, which present an increased expression of ALB [51,52]. However,

there are still few studies describing the role of ALB expression in phenotypic modulation of HSC.

The Fibrinogen Alpha Chain (FGA) and Fibrinogen Beta Chain (FGB) genes were among the downregulated genes in our analysis. According to literature, FGA and FGB were found to be upregulated during mice embryonic liver development in a study that investigated gene expression alterations of natural antisense transcripts, a type of non-coding RNA, thus revealing a possible role of these genes on regulating liver cell differentiation and liver regeneration [53]. In addiction the FGA protein was identified to be upregulated in plasma/serum of patients with chronic alcohol consumption, hepatocarcinoma and chronic hepatitis C virus infection [54] and could serve as a useful predictor for clinical progression of liver injury [55]. On the other hand, FGB is a protein closely related to the development of liver fibrosis, being a target for combined drugs against liver fibrosis [56]. Then, both FGA and FGB are important markers of hepatic fibrosis, but its role in the phenotypic modulation process of HSC is unclear.

Haptoglobin (HP) gene expression was also found to be downregulated in our DEG analysis. This gene is considered a biomarker of liver fibrosis on plasma [57]. Kempisnki et al showed positive associations between the increased serum concentrations of HP and Non-alcoholic fatty liver disease diagnosis [58]. However, it is not yet clear whether this gene acts directly on the phenotypic differentiation of HSC. The Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM1) was another gene downregulated found in our DEG analysis. PECAM1 is highly expressed in vascular cells, such as endothelial cells, where it acts in the formation of the endothelial barrier. PECAM1 plays a significant role in the adhesion cascade in injury response, the

leukocyte transmigration, the cell migration, the angiogenesis, and the cell signaling [59]. It was already shown that PECAM1 expression is upregulated in liver infected with Schistosoma japonicum, thus demonstrating an important function of this gene in the inflammatory response [60]. Nevertheless, there are no studies relating its expression to HSC modulation. It was interesting to note that some downregulated genes in qHSC and aHSC have a lack of information on their function during liver chronic diseases or HSC phenotypic modulation. This was the case for the genes Orosomucoid 1(ORM1), Orosomucoid 2 (ORM2), Apolipoprotein C1 (APOC1), and Apolipoprotein A2 (APOA2). Thus, it seems to be relevant investigating the relevance of these genes to HSC metabolism during liver fibrosis development.

HSC modulation represents a drastic change on cell phenotypic characteristics, which is associated to the cellular function of these cells in response to liver damage [1,8]. Thus, the upregulated and downregulated genes encountered in DEG analysis were subsequently classified by their Gene Ontology (GO) enrichment terms in accordance to the PANTHER database, intending to find the main pathways related to HSC function. Pathways for the upregulated genes mainly involves the organization of extracellular matrix, collagen formation, interactions between Integrin and cell surface, and assembly of collagen fibrils and other multimeric structures. Indeed, extracellular interactions between cell and extracellular matrix are known to regulate, via integrin-mediated signal transduction, the gene expression responsive for regulating cell morphology, motility, proliferation, survival, and differentiation [61–63]. In addition, integrins have already been related to promote HSC activation and migration [64]. Based in the demanding DEG analysis, we also constructed the protein-protein interaction (PPI) network. The established PPI for upregulated genes showed only one module with significant interactions that was related to the organization of extracellular

matrix, thus corroborating what was found in the pathway analysis. However, the requirement of gene expression analysis used in this study did not allow the identification of other modules, due to the criteria used in this study. In an overall view, it is possible to state that upregulated genes may have an important role in HSC activation and hepatic fibrosis establishment. Therefore, based in their significant expression, it is plausible to consider these genes as potential targets for a precise treatment for liver fibrosis.

The pathways for downregulated genes mainly involved hemostasis, platelet degranulation, and immune system response. In this way, HSC are known to play an important role in the regulation of normal hepatic parenchyma by maintaining hepatic homeostasis and promoting vasoregulation [6,8,65]. On the other hand, previous studies have shown that immune pathway modulation can lead to activation of HSC [24,66,67], hepatocarcinoma [68,69], and liver fibrosis [70,71]. Then, based on our results, the downregulated genes refer to the maintenance of the hepatic parenchyma, which is an important function of HSC. The established PPI for downregulated genes showed four modules with significant interactions; however, only two were found at the Reactome pathway database. These modules were related to the signaling by receptor tyrosine kinases and to the hemostasis pathway. Despite the few studies on the role of downregulated genes and the necessity for investigating them, it is plausible to hypothesize that these pathways could be related not only to the activation of HSC, but to the functions of quiescent HSC, which were previously described to be responsive for regulating liver blood flow at normal conditions [72].

In conclusion, here we identified the most significant DEG in the GSE68000 dataset through bioinformatics tools, based in a hard cutoff for gene expression, for

analyzing the HSC function in liver. In the frame of bioinformatics data mining approaches, this study showed that most of the top 10 upregulated or downregulated genes found in this study were described to be involved in the phenotypic modulation of HSC. However, some downregulated genes are still little known, thus indicating the need for further studies on their function in liver diseases and phenotypic transdifferentiation of HSC. Considering this premise, these genes appear to be valuable candidates for further studies, which are undoubtedly required to verify them in vitro/in vivo roles for HSC metabolism and may be important to establish new biomarkers and/or potential targets on treating fibrosis and chronic diseases of liver.

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Parte III

6 DISCUSSÃO

A fibrose hepática e a cirrose são um grande problema de saúde global, com cerca de 844 milhões de pessoas sofrendo de doença hepática crônica em todo o mundo (Marcellin and Kutala, 2018). As taxas de mortalidade por cirrose hepática continuam a aumentar, sem tratamentos antifibróticos aprovados pela "*Food and Drug Administration*" (FDA) ou pela "*European Medicines Agency*" (EMA) (Friedman et al., 2018, Koyama et al., 2016, Tapper and Parikh, 2018). Apesar do fígado ser um órgão dinâmico que participa intensamente do metabolismo do organismo, ele é propenso a lesões que podem desencadear uma resposta inflamatória aguda sem prejudicar o parênquima hepático. No entanto, se esta lesão for crônica, o dano ao parênquima hepático propicia a deposição de fibras colágenas do tipo 1, assim acarretando, ao longo do tempo, na perda de suas funções fisiológicas e, ao final, na insuficiência hepática característica da cirrose (Bataller and Brenner 2005). A etiologia da doença hepática crônica é muito complexa e envolve um amplo espectro de fatores, incluindo o estilo de vida, fatores ambientais, genes e alterações epigenéticas que influenciam a instalação da fibrose. Cabe ressaltar que até hoje não há uma terapia eficaz disponível para fibrose hepática, exceto transplante de fígado. No entanto, o estudo de novas terapia antifibróticas para atuação especifica na população de células produtoras de colágeno, em especial as HSC, é formidável para o desenvolvimento de novas terapias. Portanto, aumentar nossa compreensão dos mecanismos celulares e moleculares que regulam a fibrose hepática é fundamental para desenvolvimento de novas terapias anti-fibróticas eficazes e altamente direcionadas para pacientes com doenças hepáticas (Ramachandran and Henderson, 2016, Trautwein et al., 2015).

As HSC apresentam dois fenótipos distintos que são responsáveis pela manutenção da arquitetura hepática normal e pelas alterações teciduais responsáveis pela fibrose. Notavelmente, o fenótipo quiescente é caracterizado pela presença de gotículas perinucleares citoplasmáticas carregadas com ésteres de retinil (vitamina A). No fígado normal, as HSC quiescentes participam da vasorregulação por meio de interações de células endoteliais, da homeostase da matriz extracelular, da desintoxicação de drogas, da imunotolerância e, possivelmente, da preservação da massa de hepatócitos por meio da secreção de mitógenos, incluindo o fator de crescimento do hepatócito (HGF, do inglês *Hepatocyte growth factor*). Por outro lado, devido aos danos teciduais agudos e/ou crônicos, estas células se transdiferenciam para o fenótipo ativado. Em especial, o processo infamatório crônico resulta na ativação continua e não controlada das HSC, que culmina na deposição de MEC. Entre as características marcantes da ativação destas células estão as modificações celulares bem caracterizadas na literatura. Neste fenótipo, estas células se transdiferem em miofibroblastos contráteis que expressam alfa-actina do músculo liso, que contribui para a distorção vascular e o aumento da resistência vascular, promovendo assim a hipertensão portal. Outras características da ativação de HSC incluem a proliferação mediada por mitógenos, o aumento da fibrogênese conduzida pelo fator de crescimento do tecido conjuntivo e fator de crescimento transformador beta 1, a inflamação, a imunorregulação amplificadas e a degradação alterada da matriz (Bataller and Brenner 2005, Hernandez-Gea and Friedman 2011, Higashi, Friedman et al. 2017). Neste aspecto, o entendimento dos eventos que orquestram o processo de transdiferenciação celular são importantes para o entendimento da atuação das HSC no processo fibrótico, assim como, é compreender os eventos

importantes que ocorrem durante a fibrogênese para a análise de alternativas terapêuticas benéficas contra a fibrose hepática.

O RSV é uma molécula que tem sido amplamente estudada nos últimos 20 anos e é encontrada em vários alimentos disponíveis aos humanos, como uva e o vinho. Dentre aos potenciais efeitos biológicos do RSV, podemos destacar suas características antioxidantes, cardioprotetora anti-inflamatória, antitumoral, antimicrobianas e neuroprotetivas. Embora o RSV tenha sido amplamente estudado *in vitro* e *in vivo*, seu mecanismo de ação em todas as condições experimentais e doses permanecem indefinidos. Além disso, muitos benefícios já foram relacionados a esta molécula, principalmente à sua atividade antioxidante na proteção de órgãos como fígado, rim e cérebro contra uma variedade de danos causados pelo estresse oxidativo (Schmatz, Perreira et al. 2012). Por outro lado, na literatura atual há descrição da ação do RSV como pro-oxidante em certas condições experimentais, por exemplo em estudos de células cancerosas de pulmão (Luo, Yang et al. 2013).

Neste contexto, nosso grupo de pesquisa demonstrou em outros estudos que 24 h de tratamento de células GRX com RSV (0.1, 1 ,10 e 50 µM) foi capaz de desencadear efeitos pró-oxidantes que foram dose-dependentes, enquanto a citotoxicidade e dano celular foram demonstrados apenas na concentração de 50 µM (Martins, Coelho et al. 2014). Em outro estudo, foi evidenciado o efeito do RSV em todas as concentrações de RSV (0.1, 1 ,10 e 50 µM) no desencadeamento autofagia e mitofagia em células GRX; mas apenas os tratamentos com 1 e 10 µM causou a biogênese mitocondrial. Neste estudo também foi demonstrado a citotoxidade do tratamento de 50 µM (Meira Martins, Vieira et al. 2015). Vale ressaltar que a presente tese teve como proposito de prosseguir na linha de pesquisa que envolve o estudo do tratamento das células GRX com diferentes doses de RSV.

No capítulo 1 desta tese buscamos avaliar o efeito do resveratrol em parâmetros moleculares de ativação, capacidade de migração e na liberação de citoquinas liberadas pelas células da linhagem GRX. O tratamento utilizado foi adaptado e padronizado pelos trabalhos anteriores desta linha de pesquisa do laboratório. Desta forma, foram utilizadas as concentrações de 1 ,10 e 50 µM de RSV por um tempo máximo de 24h. Notavelmente, o tratamento na concentração de 50 µM foi capaz de aumentar a expressão proteica de GFAP, colágeno do tipo 1 e α-SMA, parâmetros de ativação amplamente descritos na literatura. No entanto, não foram observadas alterações nas concentrações 1 e 10 µM. É interessante notar, que a maior concentração de RSV utilizada neste estudo corrobora estudos anteriores que demonstraram que a concentração de 50 µM é uma concentração citotóxica, sendo um fator de estresse para estas células. Destaca-se que as proteínas analisadas são características de miofibroblastos, sendo assim consideradas, parâmetros de ativação para as HSCs.

Além disso, as análises das alterações do citoesqueleto no processo de ativação das HSC são imprescindíveis para compreender a transição fenotípica destas células. Estas respondem às demandas sistêmicas ou locais, ou seja, elas podem se converter no fenótipo necessário com profundas modificações ultraestruturais. Neste estudo, demonstramos que os tratamentos de RSV não foram capazes de alterar o citoesqueleto das GRX. Salienta-se que a linhagem GRX apresenta características morfológicas e bioquímicas de um miofibroblasto ativado.

A contração das HSC é um aspecto relevante a ser observado porque contribui para a resistência intra-hepática e hipertensão portal, que são responsáveis por grande parte da morbidade na cirrose. As HSC ativadas expressam filamentos intermediários que são associados a células musculares lisas ou esqueléticas,

incluindo α-SMA, desmina, nestrina, vimentina e isoformas de cadeia pesada de miosina de músculo esquelético (Bataller and Brenner 2005, Hernandez-Gea and Friedman 2011, Friedman and Hao 2017). Nossos resultados demonstraram que apenas o tratamento com 50 µM de RSV levou a uma menor contração do gel de colágeno devido e esse fato deve estar associado à citotoxidade do RSV nas células GRX.

Além disso, analisamos a influência do tratamento com RSV na habilidade de migração celular. Neste experimento notamos que as concentrações de RSV de 10 e 50 µM com 6 e 12 horas de tratamento foi capaz de retardar a migração das células para área lesada do gel. Interessantemente, o tratamento das células com a concentração de 50 µM de RSV obtiveram menor capacidade de migratória em todos os tempos analisados. Ressalta-se que em trabalhos anteriores do grupo de pesquisa já foi demonstrado que o tratamento com 50 µM de RSV se mostrou citotóxico. Desta maneira, a citotoxidade observada pode atuar como agente estressante que acarretou com morte por apoptose e necrose (Meira Martins, Vieira et al. 2015). A capacidade migratória das HSC é uma característica importante da sua ativação, é imprescindível para sua função biológica e pode ser influenciada pela concentração de tratamento.

As próprias HSC produzem numerosas citocinas, quimiocinas e mediadores de crescimento, que exercerem efeitos autócrinos. As citocinas/quimiocinas derivadas de HSC recrutam e ativam células inflamatórias que reagem aos mediadores por elas liberados, perpetuando a fibrogênese. O processo da fibrose hepática é um fenômeno complexo, orquestrado por uma infinidade de células, mediadores e vias de sinalização que convergem para o fenótipo ativado das HSC, desencadeando a proliferação, a migração e a deposição de MEC. Neste estudo, dosamos o Fator de Necrose Tumoral-α (TNF-α) e interleucina-10 (IL-10) em meios de cultura das células

GRX. Demonstramos que o RSV influencia a liberação destas interleucinas. Por outro lado, a secreção de interleucina 6 (IL-6) foi diminuída no meio de cultura. Estas interleucinas atuam intensamente na cascata inflamatória durante o processo da fibrose hepática. Este fato pode influenciar não apenas o destino das HSC, mas os processos imunes associados a modulação fenotípica das HSC e no processo de fibrose hepática. Vale ressaltar que neste experimento houve apenas culturas de GRX.

No capítulo 2 desta tese, buscamos analisar por meio de recursos de bioinformática os genes alvos do RSV descritos em bancos públicos. Subsequentemente, analisamos as vias de atuação destes genes e realizamos uma pesquisa bibliográfica. Utilizamos como fonte de dados o *DrugBank database,* neste banco de dados, estão inclusos as descrições de drogas e o uso de novos alvos de drogas em um servidor da *web* que serve para identificar potenciais alvos de drogas e proteínas. Neste banco de dados foram encontrados 26 genes alvos do RSV (NQO2, CSNK2A1, PTGS1, PTGS2, ALOX15, ALOX5, AHR, PI4K2B, ITGA5, ITGB3, APP, SNCA, SIRT1, ESR1, MTNR1A, MTNR1B, CLEC14A, NR1I2, NR1I3, SLC2A1, CBR1, PPARA, PPARG, AKT1, KHSRP e YARS). Destes, apenas 7 foram encontrados no banco de dados PUBMED tendo relação com a modulação fenotípica dos HSC. Além disso, a análise de rede destes genes demostrou grande interação entre os 26 genes. As vias mais significativas foram: via de transcrição do receptor nuclear (P=3.33e^{-16}), via de transcrição genérica (P=1.83e^{-07}), transcrição de RNA polimerase II (P=7.37e^{-07}), sinalização de interleucina-4 e interleucina-13 (P=1.06e^{-06}) e expressão gênica (transcrição) (P=3.01e^{-06}). Isto sugere que o RSV participa intensivamente dos processos moleculares imprescindíveis das células.

No capítulo 3 buscamos averiguar, por meio de técnicas de bioinformática, a expressão gênica de células HSC primarias humanas, onde foi analisado os genes mais diferencialmente expressos. Neste trabalho, utilizando nível de significância (P<0.001 e Log2-Fold>4), não foram encontrados genes relacionados ao RSV abordados no capítulo 2 desta tese. Desta forma, buscou-se analisar os 10 genes *upregulated* e *downregulated*. Cabe ressaltar, que apesar dos genes *upregulated* já estarem bem descritos na literatura, os genes *downregulated* ainda são pouco estudados no âmbito da transdiferenciação fenotípica das HSC.

6.1 CONCLUSÕES

Concluímos nesta tese que o tratamento com RSV foi responsável pela estimulação de vários parâmetros de ativação, e deve ter um papel na comunicação com outras células durante a fibrogênese hepática. Muitos estudos associam a ações do RSV na proteção contra doenças; contudo, em nosso modelo, a concentração de RSV foi capaz de desencadear diferentes respostas celulares. Logo, necessita-se mais trabalhos para explicar o seu papel neste modelo de HSC. Ressalta-se a importância de experimentos *in sílico* nesta pesquisa, que possibilitaram descobrir vários aspectos moleculares ainda não explorados no campo da fibrose hepática e/ou modulação fenotípica das HSC.

PERSPECTIVAS

De acordo com o apresentado nesta tese, é necessária a investigação sobre os mecanismos responsáveis tanto pela ação do RSV na modulação fenotípica das

HSC, em especial no modelo GRX, quanto no contexto da fibrose hepática. Desta forma, tem-se como perspectiva a análise com o emprego da bioinformática e as técnicas denominadas de Chip-seq, onde será possível analisar todos os genes e vias afetadas pelo tratamento com RSV. Denota-se que estas técnicas de alto rendimento serão de grande importância para o estudo e a caracterização do modelo celular GRX devido a não existência destes dados em bancos de dados públicos.

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