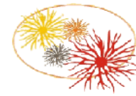


UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG

INSTITUTO DE CIÊNCIAS BIOLÓGICAS – ICB

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

Tese de Doutorado

Relação entre a aquisição da resistência a múltiplas drogas e marcadores de células-tronco em linhagens eritroleucêmicas humanas

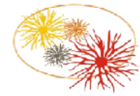
Biol. MSc. Aline Portantiolo Lettnin

Orientadora: Profª Dra Ana Paula de Souza Votto

Coorientadora: Profª Dra Gilma Santos Trindade

Rio Grande

Março de 2019



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ALINE PORTANTIOLO LETTNIN

Tese a ser apresentada como parte dos requisitos para obtenção do título de Doutora no Programa de Pós-Graduação em Ciências Fisiológicas, da Univerdade Federal do Rio Grande – FURG, sob orientação da Profª Dra Ana Paula de Souza Votto e coorientação da Profª Gilma Santos Trindade, do Instituto de Ciências Biológicas.

Rio Grande

Março de 2019

“Viva como se fosse morrer amanhã. Aprenda como se fosse viver para sempre.”

(Mahatma Gandhi)

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LISTA DE ABREVIATURAS

AA - ácido araquidônico

ABC - *ATP binding cassette* (cascata de ligação ao ATP)

ABCB1 - gene *ABCB1* pertencente a família dos transportadores ABC

ABCC1 - gene *ABCC1* pertencente a família dos transportadores ABC

ABCB1 - proteína transmembrana ABCB1

ABCC1 – proteína transmembrana ABCC1

ABL - gene *Abelson Murine Leukemia* (Leucemia murina de Abelson)

ALOX5 - gene que codifica para enzima 5-LO

BCR - gene *Breakpoint Cluster Region* (Região Cluster de Breakpoint)

***BCR-ABL* -** gene que codifica para proteína BCR-ABL

BCR-ABL - proteína resultante da fusão dos genes *BCR* e *ABL*

BLT1 - receptor de membrana de leucotrienos 1

BLT2 - receptor de membrana de leucotrienos 2

CT - Célula-Tronco

CTC - Célula-Tronco Cancerígenas

CTE - Célula-Tronco Embrionárias

CSC - *Cancer Stem Cell* (Célula-Tronco Cancerígena)

CysLT1 - receptor de membrana cistenil-LTs 1

CysLT2 - receptor membrana cistenil-LTs 2

DNR - quimioterápico Daunorubicina

FLAP - proteína ativadora da 5-lipoxigenase

GPCR - *G protein coupled transmembrane receptors* (receptores transmembrana acoplatos a proteína G)

HESC - *Human Embryonic Stem Cell* (Células-Tronco Embrionárias Humanas)

K562/ADM - linhagem celular K562 resistente ao quimioterápico Adriamicina

LMC - Leucemia Mielóide Crônica

LMA - Leucemia Mielóide Aguda

LTA4 - leucotrienos A4

LTC4 - leucotrienos C4

LTD4 - leucotrienos D4

LTE4 - leucotrienos E4

MAPKAP quinase 2 - proteína quinase 2 ativada por quinase

MDR - *Multidrug Resistance* (Fenótipo de Resistência a Multiplas Drogas)

MRP1 - *Multidrug Resistance Protein 1* (Proteína de Resistência associada a Multiplas Drogas)

mRNA - RNA mensageiro

miRNA - micro RNA

MSD0 - segmentos transmembrana 0

MSD1 - segmentos transmembrana 1

MSD2 - segmentos transmembrana 2

NDB1 – *Nucleotide Domain Binding 1* (Ligação de Domínio Nucleotídeo 1)

NDB2 - *Nucleotide Domain Binding 2* (Ligação de Domínio Nucleotídeo 2)

OCT-4 - gene que codifica para proteína OCT-4

OCT-4 - Proteína fator de transcrição de ligação ao octâmero 4

OCT4-PG - pseudogenes do gene OCT-4

PCR - *Polymerase Chain Reaction* (Reação em Cadeia da Polimerase)

POU - domínio que se liga à sequência octamer

Primer - Oligonucleotídeos iniciadores de expressão gênica

P-gp - glicoproteina P

Ph - cromossomo Philadelphia

P53 - proteína P53

SC - *Stem Cell* (Célula-Tronco)

siRNA - RNA de interferência

shRNA - gancho curto do RNA (short hairpin RNA)

TMD - *TransMembrane Domain* (Domínio TransMembrana)

VCR - quimioterápico Vincristina

5-LO - enzima 5-lipoxigenase

5-HPETE - ácido 5-hidroperoxieicosatetraenóico

5-HETE - ácido 5-hidroxieicosatetraenóico

RESUMO

A resistência a múltiplas drogas (MDR) pode ser caracterizada pela capacidade de exportar diferentes compostos químicos para o meio extracelular, sendo a principal causa na falta de sucesso na terapia do câncer. Atualmente uma possível relação do fenótipo MDR e a presença de células-tronco cancerígenas (CTCs) nesta patologia tem sido estudada. O objetivo desta tese foi investigar a relação entre a presença de células-tronco (CT), diferenciação celular e o fenótipo MDR nas linhagens celulares eritroleucêmicas humanas K562, K562-Lucena e FEPS. O silenciamento do pseudogene *OCT4-PG1* na FEPS com o plasmídeo shRNA, a sensibilidade da linhagem FEPS silenciada aos quimioterápicos vincristina (VCR) e daunorrubicina (DNR) por exclusão por azul de tripan, transformação das células K562 com concentrações crescentes de VCR e DNR, análise de expressão das proteínas ABCB1 e OCT-4 por citometria de fluxo foi realizado. Ademais, foi analisada a interação do OCT4-PG1 com transportadores pela rede STRING, atividade das proteínas de efluxo ABCB1 e ABCC1, análises de expressão gênica de *ABCB1*, *ABCC1*, *ALOX5*, *OCT-4* por PCR-tempo real nas células K562, K562-Lucena, FEPS, K562-Lucena e FEPS silenciadas para ABCB1. As células FEPS silenciadas para *OCT4-PG1* apresentaram menores níveis de mRNA e proteína OCT-4, diminuição na expressão gênica, proteica e atividade da proteína ABCB1, aumento na expressão gênica de *ALOX5* e *ABCC1*, bem como a atividade deste transportador, sensibilidade aos quimioterápicos e a rede STRING demonstrou interação direta de OCT4-PG1 com OCT-4, SOX2 e NANOG e interação indireta com os transportadores ABC. As células K562 apresentaram sensibilidade com 15, 30 e 60 nM de VCR a partir de 48h e a partir de 33,35 nM de DNR em 96h. As células K562 transformadas com os quimioterápicos VCR e DNR apresentaram aumento na expressão gênica e proteica ABCB1, diminuição na expressão gênica *ALOX5* já nas concentrações intermediárias. As células K562-Lucena silenciadas para *ABCB1* aumentaram a expressão gênica *ABCC1* enquanto este gene diminuiu na FEPS silenciadas para *ABCB1*. Ambas linhagens K562-Lucena e FEPS silenciadas para *ABCB1* aumentaram a expressão gênica de *ALOX5.* Os dados mostram que mudanças nos perfis de marcadores de células-tronco OCT-4 e ALOX5, modificaram a manutenção e aquisição do fenótipo MDR nas linhagens celulares K562-Lucena e FEPS.

Palavras-chave: Gene *ALOX5*, K562, K562-Lucena, FEPS, Proteína OCT-4, Transportadores ABCs.

ABSTRACT

Multiple drug resistance (MDR) can to be characterized by the ability to export different chemical compounds to the extracellular medium, being the main cause in the lack of success in cancer therapy. Currently a possible relationship of the MDR phenotype and the presence of cancer stem cells (CSCs) in this pathology has been studied. The aimed of this thesis was to investigate the relationship between the presence of stem cells (SC), cell differentiation and MDR phenotype in human erythroleukemic cell lines K562, K562-Lucena and FEPS. The silencing in the pseudogene *OCT4-PG1* in the FEPS line with shRNA plasmid, sensitivity of FEPS silenced to chemotherapy vincristine (VCR) and daunorubicin (DNR) (trypan blue exclusion), transformation of K562 cells with increasing concentrations of VCR and DNR, analysis of protein expression ABCB1 and OCT-4 by flow cytometry were performed. In addition, was analyzed the interaction of *OCT4-PG1* with transporters by STRING network, activity of ABCB1 and ABCC1 efflux proteins, gene expression analyzes of *ABCB1*, *ABCC1*, *ALOX5*, *OCT-4* by PCR-time real in K562, K562-Lucena, FEPS, K562-Lucena and FEPS silenced to *ABCB1* cells. *OCT4-PG1* silenced FEPS cells showed lower levels of mRNA and OCT-4 protein, decreased gene, protein expression and activity ABCB1, increased gene expression of *ALOX5* and *ABCC1*, as well as the activity of this transporter, sensitivity to chemotherapeutics and the STRING network demonstrated direct *OCT4-PG1* interaction with OCT-4, SOX2 and NANOG and indirect interaction with ABC transporters. K562 cells exhibited sensitivity at 15, 30 and 60 nM VCR from 48h and from 33.35 nM DNR in 96h. The transformed K562 cells with the chemotherapy VCR and DNR showed increase in the gene expression and protein *ABCB1*, decrease in the *ALOX5* gene expression already in the intermediate concentrations. K562-Lucena cells silenced for *ABCB1* increased the *ABCC1* gene expression while this gene decreased in the silenced FEPS cells silenced for *ABCB1*. Both lines K562-Lucena and FEPS cells silenced for *ABCB1* increased the *ALOX5* gene expression. The data show that changes in the profiles of stem cells markers, *OCT-4* and *ALOX5* modified the maintenance and acquisition of the MDR phenotype in K562-Lucena and FEPS cell lines.

Keywords: *ALOX5* gene, K562, K562-Lucena, FEPS, OCT-4 protein, ABC transporters.

INTRODUÇÃO GERAL

Heterogeneidade Tumoral

O processo de conversão de uma célula saudável em uma célula maligna ocorre a partir de várias alterações moleculares que induzem o aumento da proliferação, angiogênese, metástase, e evasão de supressão do crescimento. Diversos tipos de tumores cancerígenos tem sido desvendados na sua complexidade genética a partir de tecnologias avançadas com base em diagnósticos moleculares. Contudo, acredita-se que ao longo da sua progressão, diversos processos celulares sejam desestabilizados, agindo de forma integrada (Hanahan & Weinberg, 2011).

O câncer pode ser considerado uma doença dinâmica em que as células causadoras desta patologia sofrem alterações moleculares contínuas. Essas mudanças podem ter origem a partir de alterações genéticas (DNA), transcriptômicas (transcritos de RNAs), epigenéticas (modificações do genoma transmitidas durante as divisões celulares) ou fenotípicas (características adquiridas) que conferem às células uma heterogeneidade tumoral. Essa heterogeneidade pode ser caracterizada pela capacidade das células cancerígenas sofrerem distintas alterações moleculares e diferentes níveis de sensibilidade a terapias antineoplásicas ao longo da evolução do câncer (Dagogo-Jack & Shaw, 2017). Pode-se assim considerar que a heterogeneidade tumoral possibilita ao câncer a resistência à quimioterapia (Bhang et al., 2015; Jamal-Hanjani et al., 2017) (Figura 1).

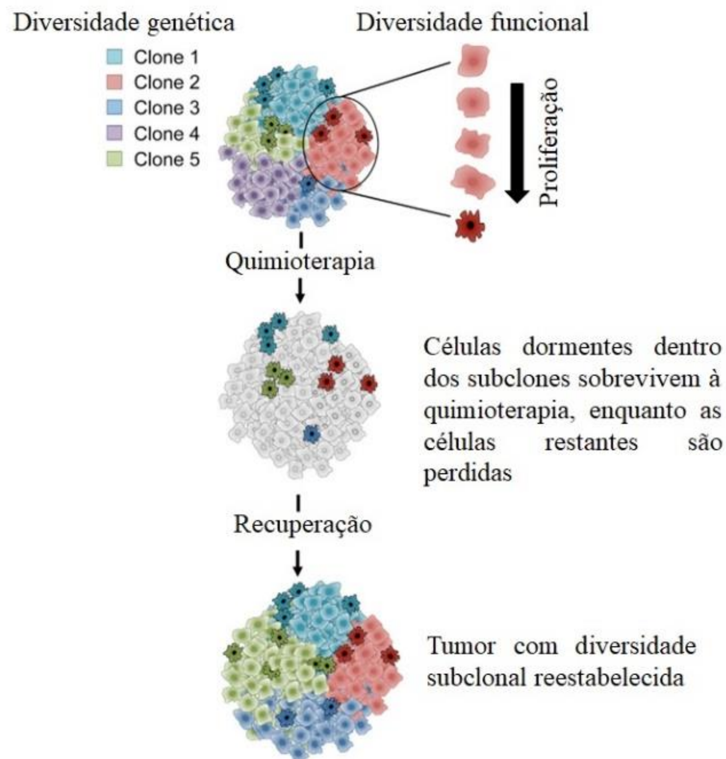

Figura 1 - Diversidade funcional entre células e impactos de resposta à terapia. Cada clone (representado pelas cores diferentes) contém uma mistura de células que variam em relação a sua capacidade proliferativa, incluindo células relativamente dormentes. Juntos, esses fatores representam a diversidade funcional presente dentro de subclones genéticos únicos. A quimioterapia pode reduzir a carga tumoral, eliminando as células altamente proliferativas nos subclones, enquanto poupa as células relativamente dormentes. Após a terapia, essas células podem semear um novo câncer. Desse modo, a diversidade subclonal pode ser alterada com a quimioterapia e pode permitir a seleção de células com mutações genéticas adicionais que conferem uma vantagem de sobrevivência. Adaptado de Kreso & Disck, 2014.

Resistência a múltiplas drogas

A resistência a múltiplas drogas (MDR) é um sistema de proteção celular capaz de ativar ou reprimir sistemas de reparo do DNA, modifica o ciclo celular, altera fases da apoptose (Baguley, 2010) e capaz de exportar diferentes compostos químicos, incluindo medicamentos com diferentes estruturas químicas e diferentes mecanismos de atividade intracelular (Stavrovskaya & Stromskaya, 2008). Os tumores que inicialmente respondem a determinados compostos, como os quimioterápicos, passam a adquirir resistência a drogas que podem ou não ser quimicamente relacionadas. Este fator torna o fenótipo MDR a principal razão para a falta de êxito no tratamento do câncer (Gottesman & Pastan,

1993; Fernandes et al., 2005; Gottesman & Ling, 2006; Ferguson et al., 2009; Fatemian et al., 2014).

Os transportadores ABC constituem uma superfamília de proteínas de membrana responsáveis pela translocação de várias substâncias, ou seja, são capazes de fazer a extrusão de compostos como produtos naturais, peptídeos e ainda, diversas drogas (Sharom et al., 2001; Ruiz et al., 2013). Este mecanismo possibilita que a concentração da droga no meio intracelular seja reduzida a um nível considerado não tóxico para a célula (Vaidya et al., 2011).

Considera-se que a superexpressão ou supressão de determinadas vias moleculares pode estar associada aos mecanismos de resistência e, consequentemente, ao fracasso no tratamento do câncer. Logo, novos estudos sobre mecanismos moleculares de resistência a drogas possibilitam a abordagem de novas vias para a resposta terapêutica (Fatemian et al., 2014).

Dentre as características do fenótipo MDR está a expressão de proteínas na membrana plasmática, com capacidade de proteger tecidos sensíveis da toxicidade endógena (Schinkel et al., 1994). Uma das proteínas mais estudadas no câncer é a glicoproteína P (P-gp/ABCB1) (Juliano & Ling, 1976; Gottesman & Pastan, 1993; Klappe et al., 2009; Celestino et al*.,* 2015), com peso molecular de 170 kDa e 1280 resíduos de aminoácidos, a qual é codificada pelo o gene MDR1 (*ABCB1*), localizado no braço longo do cromossomo 7q21 (Sonneveld & Wiemer, 1997).

A proteína ABCB1 é formada por aminoácidos organizados em duas grandes subunidades, cada uma com 1220 resíduos de aminoácidos. Cada subunidade da proteína possui um domínio citosólico, um domínio transmembrana hidrofóbico com seis alfas hélices e um domínio ligante de nucleotídeo NDB1 ou NDB2 (ligação de domínio

nucleotídeo). As porções N e C-terminal estão localizadas no citoplasma (Azzaria et al., 1989) (Figura 2).

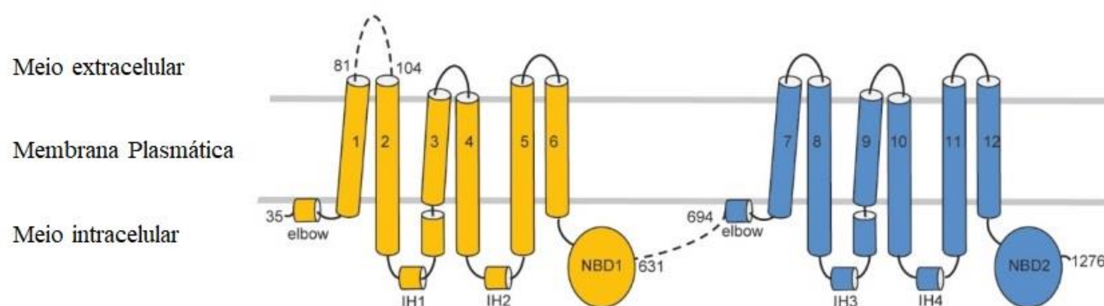

Figura 2 - Estrutura da proteína ABCB1. Diagrama esquemático de uma estrutura da glicoproteína P-gp/ABCB1. As posições de 1 a 12 são TMDs (domínio transmembrana). As estruturas NBD1 e NBD1 possuem ligação de domínio de nucleotídeo. Adaptado de Kim & Chenn et al., 2018.

De uma forma geral, a proteína ABCB1 é capaz de transportar substâncias tóxicas como xenobióticos, metabólitos, moléculas hidrofóbicas (Haimeur et al., 2004) e moléculas bioativas importantes para o desenvolvimento do tumor (Domenichini et al., 2019). Pertencente à família dos transportadores ABC (ATP-binding cassette), esta glicoproteína utiliza energia a partir da hidrólise de ATP para bombear agentes quimioterápicos para o meio extracelular (Uchiumi et al., 1993; Kosztyu et al., 2014).

Além da ABCB1, a proteína de membrana MRP1 (ABCC1), relacionada a diferentes tipos de câncer (Munoz et al., 2007), também está associada ao fenótipo MDR e pertence a família dos transportadores ABC. O gene responsável pela codificação da proteína ABCC1 está localizado no cromossomo 16, e esta possui peso molecular de 190kDa e 1531 aminoácidos (Krishnamachary & Center, 1993).

A proteína ABCC1 apresenta algumas especificidades para o transporte de moléculas para o meio extracelular, sendo capaz de realizar a extrusão de ânions orgânicos e compostos não iônicos, além de facilitar a extrusão de outros compostos como glutationa, conjugados de glutationa, co-transportados com glutationa, glucuronídeo ou

sulfato conjugados (Loe et al., 1998; Bergman 2003), esteróides, leucotrienos e prostaglandinas (Bakos & Homoloya, 2007).

Similar às demais proteínas da famíla ABC, a proteína ABCC1 possui dois TMDs (domínios transmembrana), denominados MSD1 2 MSD2, sendo que cada domínio está ligado a um NBD (NBD1 ou NBD2). Contudo, a proteína ABCC1 possui um terceiro domínio transmembranar com cinco segmentos transmembrana (MSD), o que a diferencia dos demais transportadores ABC (Cole, 2014). Ademais, apresenta as estruturas CL5 e CL7 que auxiliam na especificadade da ligação do substrato (Cole, 2014) (Figura 3).

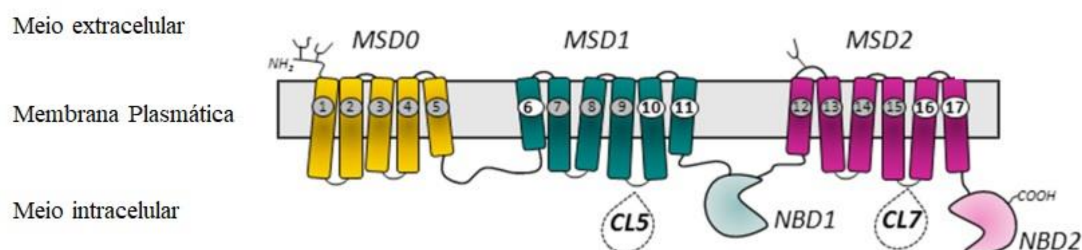

Figura 3 – Estrutura da proteína ABCC1. Diagrama esquemático de uma estrutura da proteína MRP1/ABCC1. As posições das TMD (domínio transmembrana) 6, 10, 11, 16 e 17 estão em destaque e foram identificadas como contendo os principais determinantes da especificidade do substrato MRP1. As estruturas CL5 e CL7 contêm aminoácidos envolvidos na especificidade do substrato, dobramento adequado e trafego de membrana plasmática, bem como o mecanismo de transporte do MRP1. Adaptado de Cole, 2014.

Leucemia Mieloide Crônica

O fenótipo MDR pode ser observado em diferentes tipos de câncer, como na leucemia, a qual compreende um grupo diverso de doenças caracterizado pela proliferação clonal de células cancerígenas progenitoras do sangue (Guo et al., 2014). Um dos tipos de leucemia, a leucemia mieloide crônica (LMC), é caracterizada por uma alteração nas células-tronco hematopoiéticas clonais, que apresentam uma excessiva proliferação de células progenitoras mieloides com capacidade de se diferenciar em

células mielóides, monocíticas, megacariocíticas, durante a fase estável ou crônica da doença (Vaidya et al., 2011).

A LMC também é caracterizada pela perda de características adesivas, diminuição de apoptose e presença da oncoproteína BCR-ABL (Gerber et al., 2011). Durante o diagnóstico, 95% dos pacientes apresentam o cromossomo Philadelphia (Ph) (Sawyers, 1999) formado a partir da translocação entre o gene *Abelson Murine Leukemia* (ABL), pertencente ao cromossomo 9, e o gene *Breakpoint Cluster Region* (BCR) pertencente ao cromossomo 22 (Rowley, 1973). A união desses genes forma uma mutação que produz um oncogene denominado BCR-ABL (Figura 4) , o qual torna-se responsável por sintetizar uma proteína quinase que exercerá suas funções de sinalização intracelular sem receber nenhum mecanismo regulatório (Goldman & Melo, 2003). Como consequência, a célula terá um aumento descontrolado na sua proliferação (Kurzrock et al., 1988).

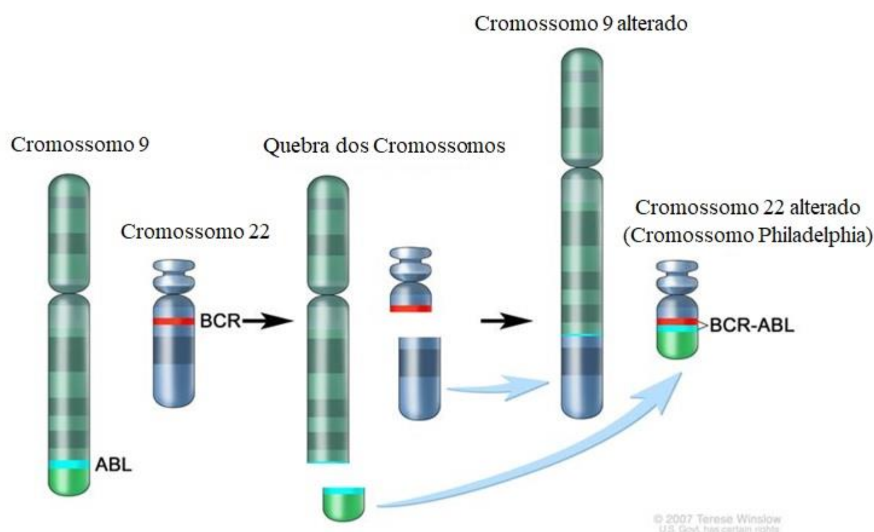

Figura 4 - Cromossomo Philadelphia. Translocação dos cromossomos 9 e 22 e a formação do oncogene BCR-ABL t(9;22). Adaptado de National Cancer Institute USA.gov. Disponível em: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/philadelphia-chromosome. Acesso em 01 de março de 2019.

Linhagens celulares com translocação BCR-ABL

As linhagens celulares estabelecidas possibilitam ampliar as pesquisas quanto à sensibilidade ao tratamento de células tumorais, considerando a resistência aos quimioterápicos e a ativação de diferentes vias e mecanismos envolvidos neste processo (Daflon-Yunes et al., 2013). Neste estudo, foram utilizadas três linhagens celulares de Leucemia Mielóide Crônica. A primeira linhagem denominada K562, a qual apresenta o cromossomo Ph, foi obtida de uma paciente com Leucemia Mielóide Crônica e apresenta potencial de diferenciação em células progenitoras precoces da medula óssea e sangue periférico (Lozzio et al., 1981). A linhagem K562 não apresenta fenótipo MDR. Buscando compreender a resistência celular aos quimioterápicos, Rumjanek e colaboradores (1994; 2001) desenvolveram uma linhagem celular leucêmica MDR denominada K562-Lucena1 (K562-Lucena) através da adição de concentrações progressivamente maiores do quimioterápico vincristina (VCR) nas células K562 Este quimioterápico desestabiliza o citoesqueleto da célula, atuando como um bloqueador da polimerização dos microtúbulos, através da ligação a dímeros de tubulina entre suas α- e β-subunidades (Himes et al., 1976; Owellen et al., 1977).

Mais recentemente, a linhagem eritroleucêmica denominada FEPS foi desenvolvida a partir da adição de concentrações crescentes do quimioterápico daunorrubicina (DNR), o qual tem como alvo celular o DNA. Esta linhagem também foi desenvolvida a partir da linhagem celular K562, apresenta características MDR e maior resistência a quimioterápicos já testados quando comparada as linhagens K562 e K562-Lucena (Daflon-Yunes et al., 2013) (Figura 5).

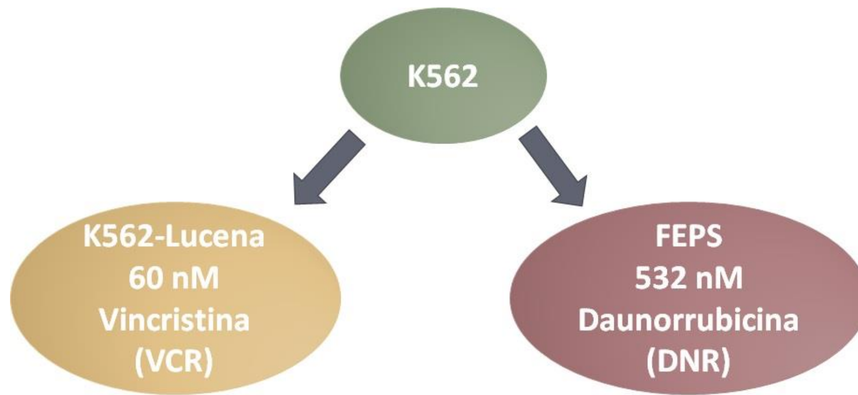

Figura 5 – Perfil comparativo das três linhagens K562, K562-Lucena e FEPS.

Comparativamente, as três linhagens K562, K562-Lucena e FEPS apresentam perfis de expressão gênica diferentes . Na comparação entre K562 e K562-Lucena foi identificada uma diferença de 130 genes, sendo 65 genes superexpressos e 65 genes pouco expressos na K562-Lucena. Quando comparado os perfis de expressão entre K562 e FEPS, foi identificado um total de 932 genes expressos diferencialmente, sendo 288 superexpressos e 644 pouco expressos na linhagem FEPS. Nos perfis de expressão entre as linhagens MDR, K562-Lucena e FEPS, foram identificados 1211 genes diferentes, sendo 459 superexpressos e 752 pouco expressos na linhagem FEPS (Moreira et al., 2014) (Tabela 1)

Na Tabela 1, Moreira e colaboradores (2014) apresentam os dez genes com as maiores diferenças de expressão em cada linhagem. O gene *ABCB1* apresentou maior diferença na expressão gênica, sendo superexpresso em ambas as linhagens celulares MDR quando comparado a linhagem K562.

Comparação experimental	Número de genes expressos diferencialmente		Os 10 genes com maior diferença na expressão
	Genes induzidos	Genes reprimidos	
K562 *vs.* Lucena 1 – VCR	65	65	*ABCB1* (+287.62); *MUC19* (+182.53); *CD36* (+159.56); *SUSD2* (−148.47); *VAMP8* (−132.79); *ITGAM* (−110.58); *DOCK8* (−109.14); *SESN3* (+96.34); *ND6* (−90.89); *IL1R1* (−75.11)
K562 *vs.* FEPS – DNR	288	644	*ABCB1* (+1759.56); *IGF1* (−1038.29); *CILP* (−1026.84); *SRGN* (−613.53); *SCIN* (+532.26); *LAPTM5* (−492.50); *ALOX5* (−454.14); *ARHGDIB* (−362.29); *VAMP8* (−362.29); *LOC283352* (−356.07)
Lucena 1 – VCR *vs.* FEPS – DNR	459	752	*ANXA1* (−1077.89); *SRGN* (−1006.41); *SCIN* (+761.66); *HBB* (+710.65); *TEX19* (−584.48); *LAPTM5* (−512.71); *LOC283352* (−501.12); *EREG* (−483.04); *IGF1* (−348.25); *CLEC2B* (−326.74)

Tabela 1. Número de genes expressos diferencialmente e os 10 genes com maior diferença de expressão por análises de microarray. As diferenças na transcrição são mostradas em parênteses em relação a primeira linhagem celular. Adaptado de Moreira et al., 2014.

Além desses genes, foi observado o perfil inverso da expressão dos genes *OCT-4, ABCB1* e *ALOX5* nas linhagens K562, K562-Lucena e FEPS (Carret-Dias et al., 2016). Em que a linhagem celular K562 apresenta baixa expressão dos genes *OCT-4* e *ABCB1*, enquanto superexpressa o gene *ALOX5*. Já as linhagens K562-Lucena e FEPS apresentam baixa expressão do gene *ALOX5* e superexpressão dos genes *OCT-4 e ABCB1* (Figura 6).

Genes	*OCT-4*	*ABCB1*	*ALOX5*
K562	Pouco expresso	Pouco expresso	Superexpresso
K562-Lucena	Superexpresso	Superexpresso	Pouco expresso
FEPS	Superexpresso	Superexpresso	Pouco expresso

Figura 6 – Diferença no perfil de expressão gênica. Quadro comparativo dos genes *OCT-4, ABCB1* e *ALOX5* nas linhagens celulares K562, K562-Lucena e FEPS.

Células-tronco Cancerígenas

Apesar da utilização de diversos tipos de quimioterápicos para combater o câncer, pesquisas sobre as células-tronco cancerígenas (CTC) tem sido realizadas para desenvolver novos tratamentos que previnem a recidiva do câncer (Nerlov 2009; Chonel & Turhan, 2011).As células-tronco (CT) apresentam como principais características a capacidade de se auto-renovar e se diferenciar em outros tipos de células que desempenham diferentes funções fisiológicas (Clarke, 2004) Ao sofrer uma alteração genética, as CT podem se tornar células-tronco cancerígenas (CTC) com capacidade de originar células cancerígenas diferenciadas (Villodre et al., 2016). Essa capacidade é possível um vez que essas células possuem caracterísitcas troncoPor outro lado uma célula somática que sofreu transformação maligna pode passar a expressar características tronco (Martinez-Climent et al., 2006), as quais podem auxiliar na manutenção do câncer.

De acordo com Reya e colaboradores (2001), no câncer, os eventos de iniciação, manutenção e crescimento de um tumor podem ser controlados pelas CTC. Uma das propriedades que diferencia as CTC das demais células tumorais é a capacidade de induzir a uma repopulação de células a longo prazo (Peitzsch et al., 2013), o que permite a essas células controlarem o início e a continuidade do tumor (Kreso & Dick, 2014). Ao longo do desenvolvimento do tumor, as CTC dão origem a diferentes subconjuntos de células, em que a maioria se distingue das suas células progenitoras. Isso torna-se possível devido a uma capacidade indefinida de diferenciação celular, possibilitando assim a formação de subpopulações de células malignas (Nguyen et al., 2012).

A predisposição em gerar novas subpopulações de CTC precisa ser considerada durante o tratamento do câncer uma vez que, após o tratamento com quimioterápicos, as subpopulações de CTC mais resistentes sobrevivem, resultando na recidiva do tumor. Assim, pode-se considerar que estas células sofrem alterações genômicas e epigenômicas

contínuas, que possibilitam desenvolver resistência ao tratamento, sendo essas características transmitidas para as próximas gerações de CTC (Magee et al., 2012). Alguns autores sugerem que esta resistência é adquirida em resposta ao tratamento (Stratton, 2011), enquanto outros sugerem que as CTC apresentam uma resistência intrínseca à terapia antineoplásica, o que as tornam capazes de iniciar a recaída e a progressão do câncer (Cojoc et al., 2015). Segundo Dean e colaboradores (2005), é possível encontrar altos níveis de expressão de transportadores ABC específicos em CT, os quais estão diretamente relacionados ao fenótipo MDR.

Dentre algumas características, as CTC apresentam plasticidade epigenética (capacidade de modificar a expressão dos genes, sem alterar as sequências de bases do DNA) e evolução genética (alteração na frequência dos alelos de um ou um conjunto de genes, ao longo das gerações) (Cojoc et al., 2015). Uma das dificuldades enfrentadas no tratamento eficaz para eliminação do câncer consiste na heterogeneidade do tumor resultante de uma diversidade de CTCs. Dessa forma, é preciso considerar e avaliar a evolução do câncer a partir de duas vias:

(1) evolução linear, em que CT sofrem sucessivas mutações e geram clones que mantem as mutações das células ancestrais, possibilitando o crescimento ou sobreviência do tumor.

(2) Outra possível via é a evolução ramificada, em que se propagam subclones de populações de células tumorais que compartilham o mesmo ancestral comum (Figura 7). Dentre as duas formas, a evolução ramificada possibilita mais oportunidades para desenvolver um tumor heterogêneo (Hiley et al., 2014; Dagogo-Jack & Shaw, 2017).

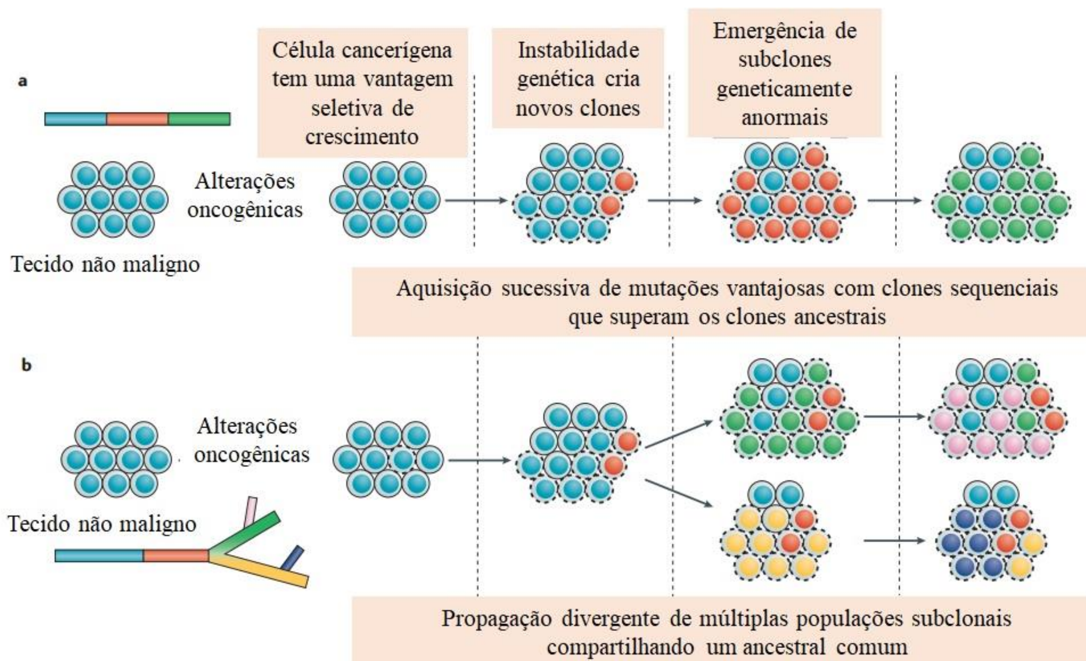

Figura 7 - Distinção entre evolução tumoral linear e ramificada. Dois grandes padrões de evolução existem dentro do contexto da evolução clonal. a) Modelo de evolução linear: alterações genéticas sequenciais conferem uma vantagem de forma que as sucessivas gerações (vermelho, seguido de verde) sejam capazes de superar os clones anteriores (azul), que não possuem essa vantagem de adequação. A sobrevivência de clones dominantes (verde) protege as mutações do ancestral. b) Modelo de evolução ramificada: múltiplas populações geneticamente distintas (verde, rosa, amarelo, roxo) emergem de um clone ancestral comum (vermelho), com certas populações subclonais divergindo do ancestral comum. Adaptado de Dagogo-Jack & Shaw, 2017.

Marcadores de célula-tronco cancerígena: relação com o câncer e fenótipo MDR

As CT possuem diversos marcadores que desempenham funções específicas para manutenção das características tronco. Relacionados a pluripotência das CT, as proteínas OCT-4, SOX2 e NANOG são fatores de transcrição (regulam a expressão de determinados genes) e formam uma rede de transcritos importantes para a divisão de CT indefinidas, mas sem alterar carcaterísticas como auto-renovação e diferenciação celular (Atari et al., 2011).

A proteína OCT-4 atua desempenhando um importante papel como regulador chave da pluripotência nas primeiras fases do desenvolvimento embrionário de CT

(Brehm et al., 1997). Também conhecido como *OCT-3* ou *POU5F1*, o gene *OCT-4* em humanos está localizado na posição p21.3 do cromossomo 6 (Wang & Dai, 2010), e apresenta um domínio POU com habilidade para ligar a uma sequência octâmera (ATGCAAAT) (Scholer et al., 1989). Dentre outras atribuições, o gene *OCT-4* atua como marcador específico para totipotência (Deyev & Polanovsky, 2004); determina a síntese de proteínas transmembrana como ABCG2 (Chen et al., 2008); mantem a auto renovação de CT em murinos (Cavaleri & Scholer, 2003); define a diferenciação, desdiferenciação ou auto renovação de CT embrionárias (CTE) (Niwa et al., 2000) e induz a pluripotência em fibroblastos humanos (Salci et al., 2015).

Em humanos, o gene *OCT-4* pode codificar três diferentes isoformas de transcrito, denominadas OCT4A, OCT4B e OCT4B1 (Wang & Dai, 2010). A isoforma OCT4A está localizada principalmente no núcleo das células, conferindo características como pluripotência e autorenovação de CT embrionárias. Por outro lado, o OCT4B está presente principalmente no citoplasma de células somáticas cancerígenas, não sendo capaz de influenciar na autorenovação de CT (Cauffman et al., 2006; Lee et al., 2006). Quanto à isoforma OCT4B1, pode ser regulada pelas vias de indução de diferenciação celular, sendo super expressa em CT e CTC (Atlasi et al., 2008). Gazouli et al. (2012) verificaram que a isoforma OCT4B1 pode representar um potente biomarcador para a iniciação, progressão e diferenciação em câncer colorretal.

Como mencionado anteriormente, o fenótipo MDR é expresso na linhagem celular leucêmica K562-Lucena, mas ausente na sua linhagem parental K562. Marques et al., (2010) constataram que ambas as linhagens apresentam fenótipo $CD34^{+}CD38^{-}$ o que é considerado um marcador de célula tronco hematopoiética. Este fenótipo caracteriza a presença de CT hematopoiéticas nestas linhagens. Os autores ainda observaram que regiões promotoras dos genes *ABCB1*, *ABCC1* e *ABCG2* (sintetiza a proteína

transmembrana BCRP/ ABCG2) contem sítios de ligação que interagem com o fator de transcrição OCT-4, o qual é considerado um marcador de CTC.

Oliveira et al. (2014) constataram, a partir do sequenciamento da região de domínio homeobox (sequência de pares de base no DNA que permite a ligação de uma proteína) do gene *OCT-4* em linhagens de LMC, que este fator de transcrição é um alvo direto de mutações e estas mutações podem contribuir para o fenótipo MDR.

Apesar da capacidade de regular diferentes vias de expressão gênica na célula, a expressão do gene *OCT-4* pode ser regulada por pseudogenes. Apesar da semelhança, os seis pseudogenes estão localizados em cromossomos diferentes do gene parental (Suo et al., 2005). O gene *OCT-4* apresenta sequências genômicas de nucleotídeos com seis pseudogenes denominados *OCT4-PG1*, *OCT4-PG2*, *OCT4-PG3*, *OCT4-PG4*, *OCT4-PG5*, e *OCT4-PG6* (Pain et al., 2005).

Os pseudogenes são considerados semelhantes aos seus genes parentais, contudo, apresentam sequências não funcionais do DNA que expressam RNAs não-codificantes com padrões diferentes de seus genes ancestrais (Balakirev & Ayala, 2003). Em outras palavras, os pseudogenes podem apresentar códons de parada prematura, inserção ou deleção de nucleotídeos e mutação Frameshift que anulam a tradução em proteínas (Poliseno et al., 2010), assim o RNA transcrito será lido de forma errada pelo ribossomo impossibilitando a tradução em proteínas funcionais.

Devido a incapacidade de sintetizar uma proteína funcional, os pseudogenes podem atuar na célula ativando ou inibindo a síntese da proteína do gene parental a partir de uma regulação pós-transcricional em que o miRNA (RNA não codificante) do pseudogene pareia com o mRNA do gene parental regulando sua estabilidade e tradução (Muro et al., 2011)Ademais, os pseudogenes podem regular a expressão do gene parental a nível epigenético competindo com outros miRNA que se ligam ao gene parental,

regulação pós transcricional através de siRNA (RNA de interferência) e ainda regular a expressão de mRNA de genes não relacionados através de esiRNA (Figura 8) O silenciamento gênico pós-transcricional consiste na inserção de um siRNA sintético de fita dupla no meio intracelular. Este siRNA pareia com o mRNA de interesse e degrada o mesmo, inibindo assim a síntese final de uma proteína.

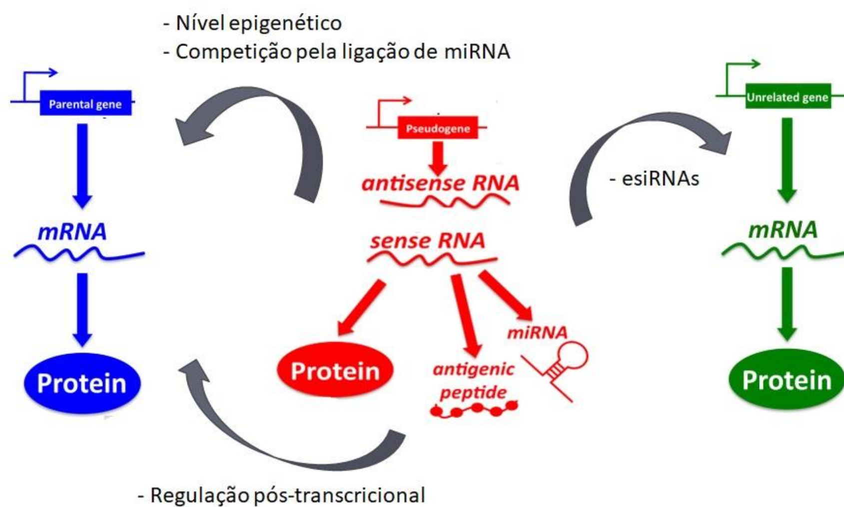

Figura 8 – Diferentes funções dos pseudogenes. Adaptado de Poliseno et al., 2015.

Em experimentos com a linhagem celular K562-Lucena, Carrett-Dias (2015) verificou que o silenciamento do pseudogene *OCT4-PG1* diminuiu a expressão do gene *ABCB1*. Segundo Suo et al. (2005), a transcrição de *OCT4-PG1* ocorre em tecidos e linhagens celulares cancerígenas, mas não em células de carcinoma embrionário, fibroblastos e tecidos normais. Ademais, a transcrição do pseudogene *OCT4-PG1* no câncer pode estar envolvida na regulação da atividade do gene *OCT-4*, sendo assim relevante a sua pesquisa para a carcinogênese.

Dentre as propriedades das CT, a diferenciação celular consiste na capacidade de se diferenciar e dar origem a qualquer tipo de célula com diferentes funções no organismo

(Clarke, 2004) Um dos marcadores das CT que está relacionado a indiferenciação celular é o gene *ALOX5*, o qual é formado por 14 exons e está localizado na região 10q11 do cromossomo 21. Na região promotora do gene estão localizadas sequências GC com sítios de ligação para fatores de transcrição como SP1, EGR-1 e NF-κB (El-Deiry et al., 1992), os quais podem regular a expressão do gene *ALOX5*.

Além das regulações do gene *ALOX5* em CT, o mesmo pode estar envolvido em diversos processos fisiológicos e patológicos, como a inflamação (Chen et al., 1994; Yokomizo et al., 2001). Este gene é responsável por codificar para a síntese da proteína 5-lipoxigenase (5-LO), a qual atua no citoplasma das células e possui como principal função a produção de leucotrienos, um grupo de mediadores lipídicos pró-inflamatórios derivados da quebra do ácido araquidônico (AA) (Samuelsson, 1983).

Em uma célula cancerígena, a via de sinalização para *ALOX5* tem início pela mediação e ativação da proteína MAPKAP quinase 2 responsável pela translocação da enzima 5-LO pela membrana nuclear (Flamand et al., 2009). Na membrana nuclear, a enzima 5-LO é ativada por FLAP (proteína de ativação de 5-LO) que permite o metabolismo do ácido araquidônico (AA) (Mancini et al., 1993; Abramovitz et al., 1993). Este, será transformado em ácido 5-hidroperoxieicosatetraenóico (5-HPETE), reduzido a ácido 5-hidroxieicosatetraenóico (5-HETE) por glutationa peroxidase (GPx), oxidado pela enzima microssomal 5-HEDH em 5-oxo-ETE, um potente quimioatraente de eosinófilos (Schwenk & Schroder 1995; Rokach & Powell, 2005) ou ainda pode ser convertido em LTA4 (leucotrienos) (Samuelsson & Funk, 1989) (Figura 9).

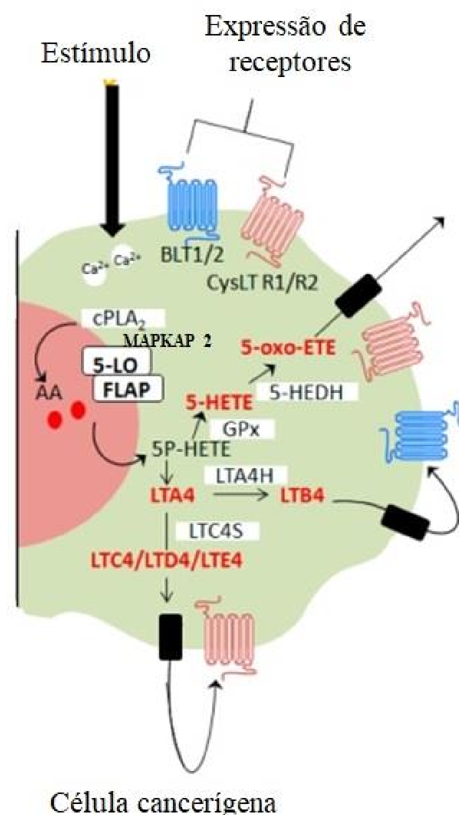

Figura 9 - Via de sinalização da enzima 5-LO em célula cancerígena. MAPKAP quinase 2 - translocação da enzima 5-LO pela membrana nuclear; FLAP -proteína de ativação de 5-LO; 5-HPETE - ácido 5-hidroperoxieicosatetraenóico; 5-HETE - ácido 5-hidroxieicosatetraenóico; 5-oxo-ETE - ácido 5-hidroxieicosatetraenóico oxidado; 5-HEDH - enzima microssomal; GPx - glutationa peroxidase; LTA4 – leucotrienos A4. Adaptado de Moore & Pidgeon, 2017.

Os leucotrienos produzidos a partir da via de sinalização oriunda pela ação da enzima 5-LO, são denominados LTC4, LTD4 e LTE4. Estes subtipos de leucotrienos podem sinalizar na célula de forma autócrina e parácrina através da ligação com receptores transmembrana chamados GPCR, que estão acoplados a proteína G. Os receptores CysLT-R1 e CysLT-R2 (cistenil-LTs-R) são sinalizados pelo influxo de cálcio e os receptores BLT1 e BLT2 são ativados pelo leucotrieno LTB4 induzindo ao influxo de cálcio e a inibição de adenolato ciclase (Peres et al., 2007). Essa via de sinalização na célula cancerígena mantém a produção ativa de leucotrienos, importante para o processo inflamatório.

O gene *ALOX5* é superexpresso em CT de ratos com leucemia mielóide crônia, atua na regulação da função de CT leucêmicas e pode ter sua expressão regulada pela proteína BCR-ABL (Chen et al., 2009). Contudo, o silenciamento do gene *ALOX5,* utilizando um plasmídeo recombinante shRNA-Alox5, diminui significativamente os níveis do gene *BCR-ABL* e os níveis da proteína de fusão BCR-ABL, além de aumentar as taxas de apoptose nas células K562/ADM (Luo et al., 2017). Isso é possível, uma vez que, a proteína BCR-ABL regula a expressão do gene *ALOX5*, dessa forma, a deficiência ou inibição do gene *ALOX5* previne o início da leucemia induzida por BCR-ABL e pode influenciar na diferenciação, divisão celular e sobrevivência das CT (Chen et al., 2009).

A inibição da enzima 5-LO em CT de glioma, quando tratadas com o composto Nordy (ácido dl-nordihydroguaiaretic sintético), inibiu a auto-renovação e induziu a diferenciação celular (Wang et al., 2011). Entretanto, a função do gene *ALOX5* parece ser específica para algumas leucemias, visto que células HL60 indiferenciadas, linhagem celular de leucemia promielocítica, expressam baixos níveis do gene *ALOX5* (Ponton et al., 1997). Já em leucemia mielóide crônica, o gene *ALOX5* é superexpresso na linhagem celular K562 (não MDR), e pouco expresso nas linhagens K562-Lucena e FEPS, ambas com o fenótipo MDR. Dessa forma, torna-se relevante avaliar a relação entre marcadores de CT e fenótipo MDR em linhagens eritroleucêmicas humanas.

OBJETIVO GERAL

Investigar a relação entre marcadores de células-tronco, marcadores de diferenciação celular e o fenótipo de resistência a múltiplas drogas (MDR) em células eritroleucêmicas humanas.

OBJETIVOS ESPECÍFICOS

- ✓ Avaliar a influência do pseudogene *OCT4-PG1* sobre o marcador de células-tronco *OCT-4*, sobre a expressão de *ALOX5*, sobre a expressão e atividade dos transportadores *ABCB1* e *ABCC1*, bem como na sensibilidade da linhagem FEPS aos quimioterápicos VCR e DNR.
- ✓ Avaliar a relação entre a superexpressão de *ABCB1* e a expressão de *ALOX5* nas linhagens K562, K562-Lucena e FEPS.

CAPÍTULO I

SILENCING THE OCT4-PG1 PSEUDOGENE REDUCES OCT-4 PROTEIN LEVES AND CHANGES CHARACTERISTICS OF THE MULTIDRUG RESISTANCE PHENOTYPE IN CHRONIC MYELOID LEUKEMIA

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ORIGINAL ARTICLE

Silencing the *OCT4-PG1* pseudogene reduces OCT-4 protein levels and changes characteristics of the multidrug resistance phenotype in chronic myeloid leukemia

Aline Portantiolo Lettnin[1,2] · Eduardo Felipe Wagner[2] · Michele Carrett-Dias[1] · Karina dos Santos Machado[3] · Adriano Werhli[3] · Andrés Delgado Cañedo[4] · Gilma Santos Trindade[1] · Ana Paula de Souza Votto[1,2,5]

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Abstract
Cancer stem cells show epigenetic plasticity and intrinsic resistance to anti-cancer therapy, rendering capable of initiating cancer relapse and progression. Transcription factor OCT-4 regulates various pathways in stem cells, but its expression can be regulated by pseudogenes. This work evaluated how *OCT4-PG1* pseudogene can affect OCT-4 expression and mechanisms related to the multidrug resistance (MDR) phenotype in FEPS cells. Considering that OCT-4 protein is a transcription factor that regulates expression of ABC transporters, level of gene expression, activity of ABC proteins and cell sensitivity to chemotherapy were evaluated after *OCT4-PG1* silencing. Besides we set up a STRING network. Results showed that after *OCT4-PG1* silencing, cells expressed OCT-4 gene and protein to a lesser extent than mock cells. The gene and protein expression of *ABCB1*, as well as its activity were reduced. On the other hand, *ALOX5* and *ABCC1* genes was increased even as the activity of this transporter. Moreover, the silencing cells become sensitive to two chemotherapics tested. The network structure demonstrated that OCT4-PG1 protein interacts directly with OCT-4, SOX2, and NANOG and indirectly with ABC transporters. We conclude that *OCT4-PG1* pseudogene plays a key role in the regulation OCT-4 transcription factor, which alters MDR phenotype in the FEPS cell line.

Keywords ABC transporters · Cancer stem cells · MRP1 protein · P-glycoprotein · STRING database

Abbreviations

CFDA	Carboxy fluorescein diacetate
SC	Stem cells
CSC	Cancer stem cells
DNR	Daunorubicin
hESCs	Human embryonic stem cells
INDO	Indomethacin
MDR	Multidrug resistance
MRP1	Multidrug resistance associated protein
P-gp	P-glycoprotein
Rho 123	Rhodamine 123
VP	Verapamil

✉ Ana Paula de Souza Votto
anavotto@yahoo.com.br; anavotto@furg.br

1 Post-Graduate Program in Physiological Sciences - PPGCF, Federal University of Rio Grande -FURG, Avenue Itália, Km 8, Rio Grande, RS Zip Code 96203-900, Brazil

2 Laboratory of Cell Culture, Institute of Biological Sciences - ICB, Federal University of Rio Grande -FURG, Avenue Itália, Km 8, Rio Grande, RS Zip Code 96203-900, Brazil

3 Center of Computational Sciences - C3, Federal University of Rio Grande -FURG, Avenue Itália, Km 8, Rio Grande, RS Zip Code 96203-900, Brazil

4 Federal University of Pampa - UNIPAMPA, Avenue Antônio Trilha, 1847, São Gabriel, RS Zip Code 97300-000, Brazil

5 Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Avenida Itália, Km 8, s/n, Rio Grande, RS Zip Code 96203-900, Brazil

Introduction

A key feature of stem cells (SC) is their ability to self-renew and differentiate into other types of cells, which perform different physiological functions. With some similarities to SC, cancer stem cells (CSCs) can give rise to differentiated cancer cells due to certain markers they share with normal stem cells [1]. In cancer, the events of initiation, maintenance, and growth of a tumor can be controlled for CSCs [2]. Moreover, these cells can induce long-term repopulation, which differentiates CSCs from

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the bulk of tumor cells [3] and enables them to initiate and maintain tumor development [4]. The CSCs show epigenetic plasticity, genetic evolution, and an intrinsic resistance to anticancer therapy, properties that render them capable of initiating cancer relapse and progression [5].

Within the group of SC, embryonic stem cells are considered an excellent experimental model for understanding one challenge of developmental cell biology: how combinations of transcription factors control gene expression [6]. During the early stages of embryonic stem cell development, the transcription factor OCT-4 acts as a key regulator of pluripotency [7]. The *OCT-4* gene in humans is located at the p21.3 position of chromosome 6 [8] and has a POU domain capable of binding to an octamer sequence (ATGCAAAT) [9]. The OCT-4 protein, encoded by the founding POU5 class member POU5F1, is expressed in pluripotent cells such as embryonic stem cells and inner cell mass [10]. Considering the POU domain, OCT-4 has many regulatory functions, e.g., maintenance of embryonic stem cells [11]; self-renewal of murine stem cells [12]; acting as a specific marker of totipotency [13]; alteration of expression pathways of the ABCG2 gene [14] and induction of pluripotency in human fibroblasts [15].

In chronic myeloid leukemia, Marques and et al. [16] observed in the K562 and K562-Lucena1 lineages that the promoter regions of the *ABCB1, ABCC1* and *ABCG2* genes contain binding sites that interact with the transcription factor OCT-4, which is considered a marker of CSCs. It is possible to find high levels of expression of specific ABC transporters in SC, which are directly related to the multidrug resistance (MDR) phenotype [17]. This phenotype can be found in different types of cancer, such as leukemia, which comprises a diverse group of diseases characterized by the clonal proliferation of blood progenitor cancerous cells [18]. Among the four types of leukemia, chronic myeloid leukemia is characterized by a change in clonal hematopoietic stem cells, which present excessive proliferation of myeloid progenitor cells capable of differentiation during the stable or chronic phase of the disease [19].

Proteins of the ABC family of transporters play important roles in transporting toxic substances, such as xenobiotics and their metabolites, across cell membranes [20]. Among the various proteins of the ABC family, the major ABC proteins investigated as regulators of the MDR phenotype are MDR1 (P-glycoprotein [P-gp], ABCB1), MRP1 (multidrug resistance-associated protein 1, ABCC1) and BCRP (ABCG2, breast cancer resistance protein) [20–22]. In tumor cells, the ATP-binding cassette (ABC) superfamily is highly expressed, and this effect is associated with to multidrug resistance [20]. Therefore, the MDR phenotype could be related to the ability of tumor cells to resist various chemotherapy drugs with different structures and cell targets [23].

Tumors that respond to certain medicines, such as chemotherapeutic agents, become resistant to various drugs that may or not be chemically related. This property of multidrug resistance is the main reason for cancer treatment failure [24–27]. The overexpression or suppression of certain molecular pathways may be associated with resistance mechanisms; thus, knowledge about molecular mechanisms related to cancer makes it possible to approach new pathways to achieving a therapeutic response [27].

Despite its ability to regulate various pathways in the cell, expression of the *OCT-4* gene can be regulated by pseudogenes [28]. For the *OCT-4* gene, analysis of genomic nucleotide sequences proposed six pseudogenes designated *OCT4-PG1, OCT4-PG2, OCT4-PG3, OCT4-PG4, OCT4-PG5*, and *OCT4-PG6* [29]. Genes with genomic DNA sequences similar to those of normal genes [30] but with nonfunctional copies that do not produce full-length proteins are classified as pseudogenes [31]. However, pseudogenes can be related to the pathogenesis of many diseases due to their capacity to regulate their parent genes, tumor suppressors, and oncogenes [31, 32]. According Muro et al. [33] it is possible that pseudogenes can to regulate their parental gene using miRNAs as post-transcriptional regulators. The pseudogene OCT4-PG4 was able to regulate the level of OCT4 protein through the expression of non-coding RNA in hepatocellular carcinoma [34]. The *OCT4-PG1* pseudogene is transcribed in cancerous cell lines and tissues but not in embryonic carcinoma cells, fibroblasts, and normal tissues. Transcription of the *OCT4-PG1* pseudogene in cancer may be involved in the regulation of *OCT-4* gene activity, thus being relevant to carcinogenesis [28].

As a means of understanding some of the mechanisms of resistance in chronic myeloid leukemia, the present work aimed to evaluate how the *OCT4-PG1* pseudogene can affect the OCT-4 protein and mechanisms related to the MDR phenotype.

Materials and methods

Cell and culture condition

FEPS cell lines were obtained from the Tumoral Immunology Laboratory at the Medical Biochemistry Institute of the Federal University of Rio de Janeiro (Brazil). FEPS cells were developed by exposing K562 cells, to increasing concentrations of daunorubicin hydrochloride (DNR) (Sigma-Aldrich, Brazil) [35]. The cells were grown in RPMI1640 (Gibco, USA) medium supplemented with sodium bicarbonate (2.0 g/L) (Vetec, Brazil), L-glutamine (0.3 g/L) (Vetec, Brazil), 10% fetal bovine serum (Gibco, Brazil), 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 μg/mL] and amphotericin B [0.25 μg/mL]) (Gibco,

Table 1 Primers sequences used in gene expression analysis

Gene	Primers sequence 5′–3′	Primers Efficiency (%)	GenBank accession number
OCT-4	F: TTCCCCATGGCGGGACACC R: CCCCTGGCCCATCACCTCC	96,09	NM_002701
OCT4-PG1	F: ATGCTTCAGGCACTGTGTTC R: TGTGACCGTATGGCTGTGTG	100,33	NM_001159542.1
ABCB1	F: CCTCAGTCAAGTTCAGAGTCTTCA R: CTCCACTTGATGATGTCTCTCACT	102,01	NM_000927
ABCC1	F: GGATCTCTCCAGCCGAAGTCT R: GTGATGGGAGCCAGAAGCA	99,25	XM_017023237.1
ALOX5	F: GTGGCGCGGTGGATTC R: TGGATCTCGCCCAGTTCCT	94,99	XM_011539564
βACTIN	F: CCACCCCACTTCTCTCTAAGGA R: ACCTCCCCTGTGTGGACTTG	103,38	NM_001101
EF1α	F: GCCAGTGGAACCACGCTGCT R: ATCCTGGAGAGGCAGGCGCA	103,14	NM_001402
B2M	F: CTCACGTCATCCAGCAGAGAA R: TCGGATGGATGAAACCCAGAC	98,19	NM_004048.2

OCT4 POU class 5 homeobox 1 (POU5F1) transcription factor, *OCT4-PG1* POU class 5 homeobox 1B (POU5F1B) pseudogene, *ABCB1* TP-binding cassette, sub-family B (MDR/TAP), *ABCC1* ATP binding cassette, subfamily C (MRP1), *ALOX5* arachidonate 5-lipoxygenase (5-LO), *βACTIN* beta actin, *EF1α* eukaryotic translation elongation factor 1 alpha 1, *B2M* beta-2-microglobulin

USA). FEPS cells were maintained at a concentration of 2×10^4 cells/mL in 24 well culture plates at 37 °C in a 5% CO_2 humidified environment.

Plasmid

The POU5F1B SureSilencing shRNA Plasmid (Qiagen, cat. no. KH66786) contains neomycin resistance gene. The plasmids were first transformed into competent *E. coli* cells (Top10 strains), selected with ampicillin, and purified with the Plasmid Maxi-Prep Kit (Qiagen, Germany). Plasmid preparations were quantified by fluorometry in a Qubit™ fluorometer, using Quanti-iT™ dsDNA Br Assay Kit (Invitrogen, USA). The result was confirmed by electrophoresis in 1.5% agarose gel with ethidium bromide (0.5 µg/mL).

POU5F1B silencing

For gene silencing was used the POU5F1B shRNA plasmid (Qiagen, cat. no. KH66786). The FEPS cells were silenced for the *POU5F1B* gene expression by transfection with shRNA plasmids provided in the Qiagen SureSilencing kit (QIAGEN™, Dusseldorf, Germany). The 1×10^6 cells were transfected with 1.2 µg/mL of the POU5F1B shRNA and mock shRNA plasmids, considered as control, using a Gene Pulser Xcell™ Electroporation Systems according Delgado-Cañedo et al. [36]. Immediately, cells were incubated in a well of a 24-well microplate at 37 °C and 5% CO2. After 24 h of transfection, 1 mg/ml of G418 (Sigma Chemical Co., St. Louis, USA) was added for selection of successfully transfected cells. After fifteen days, G418 concentration was kept at 0.5 mg/ml in cell culture. To confirm gene silencing, levels of expression of the *OCT4-PG1* pseudogene were evaluated by Real-time PCR in POU5F1B shRNA (silenced cells) and in Mock shRNA (control cells).

Gene expression analysis

Total RNA extraction and cDNA synthesis

Total RNA was extracted from six samples of each line cell with an amount of 2×10^4 per sample cells according to the protocol of the manufacturer of TRIzol Reagent (Invitrogen, Brazil). The quality and quantity of RNAs was measured by BioDrop µLite spectrophotometer (BioDrop, England). The RNA integrity was confirmed by electrophoresis in 1.5% agarose gel with ethidium bromide (0.5 µg/mL). For cDNA synthesis was performed by reverse transcription of 1 µg RNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Brazil).

Gene expression

The gene expression analysis was performed using Real-time PCR System 7300 equipment (Applied Biosystems, Brazil). PCR reactions were carried out using Gotaq qPCR Master Mix (Promega Corporation, Brazil). Gene-specific primers (Table 1) were designed based on sequences available

in GenBank using the Primer Blast tool (http://www.ncbi.nlm.nih.gov). Previously, the PCR amplification efficiency of each primers pair was evaluated by serial dilutions reactions where the efficiency of reactions showed appropriate parameters (Table 1). The *EF1α, B2M* and *βACTIN* genes were choosen as reference genes after presented stability when tested with geNorm applet [37]. Normalization factor was calculated as the geometric mean of the expression values of the reference genes tested by geNorm applet. Relative expression levels of the target genes are calculated by dividing the expression value of the target gene by the normalization factor.

Analysis of protein expression by flow cytometry

FEPS silenced and mock cells in the concentration of 1×10^6 cells/mL were fixed with 200 µL BioLegend´s Nuclear Factor Fixation Buffer (BioLegend, San Diego, USA), at room temperature in the dark for 60 min. Samples were centrifuged in 300–400 XG for 5 min at room temperature and vortexed to loosen the pellet. It was washed with 200 µL BioLegend's Nuclear Factor Permeabilization Buffer for thrice. After washed, was add 3 µL of flurochrome-conjugated antibody Alexa Fluor® 488 anti-Oct4 (Oct3) mouse IgG2b, κ Clone 3A2A20 (BioLegend) for OCT-4 protein expression and add 3 µL of FITC mouse anti-human ABCB1 clone 17f9 (Becton Dickison and Company, Franklin Lakes, USA) for ABCB1 protein expression. In each sample and cells were incubated at room temperature in the dark for 30 min. After incubation, samples were centrifuged and washed with 200 µL BioLegend´s Nuclear Factor Permeabilization Buffer for thrice. Lastly, cells were washed in 200 µL of PBS and analyzed by flow cytometer.

Analysis of the activity of ABC transporters

The ABCB1 and ABCC1 transport activity were measured using fluorescents Rhodamine 123 (Rho 123) and Carboxy Fluorescein Diacetate (CFDA) both from Sigma (Sigma Chemical Co., St. Louis, USA). The cells (2×10^5 cells/mL) were centrifuged (197 XG for 2 min), washed with PBS and fresh medium, containing 300 ng/mL of Rho 123 or 230 µg/mL of CFDA, was added. The cells were incubated for 30 min at 37 °C in the atmosphere of 5% CO2, washed once in PBS and left to extrude the dye in dye-free medium for another 30 min at 37 °C. These incubations were performed in the presence or absence of ABCB1 inhibitors VP (10 µM) and ABCC1 inhibitors INDO (300 µM) (Sigma Chemical Co., St. Louis, USA). Lastly, the cells were washed once and suspended in PBS for determination excitation and emission wavelength (485/590 nm) using fluorimeter (Filter Marx F5, Molecular Devices, USA). The ABCC1 protein activity also was analyzed in the presence or absence of ABCB1 inhibitors MK571 (50 µM) by flow cytometry.

Sensitivity of silenced cell line to DNR and VCR

FEPS silenced and mock cells were centrifuged, suspended in RPMI 1640 medium and maintained in 96-well culture plates to a final concentration of 2×10^4 cells/mL. To evaluate the MDR phenotype modulation, FEPS cells were treated with 532 nM of the DNR or 60 nM of VCR chemotherapic (Sigma Chemical Co., St. Louis, USA). The number of viable cells was assessed by trypan blue (Gibco, USA) exclusion assay 72 h after incubation.

STRING database

STRING is a database that integrates consolidating known and predicted protein–protein interactions for a large number of organisms. The interactions include direct (physical) as well as indirect (functional) associations. The STRING[1] database contains data from different sources including available experimental data, known pathways and protein complexes from curated databases and computational predicted information derived from systematic co-expression analysis, automated text-mining of the scientific literature and computational transfer of interaction knowledge between organisms based on gene onthology.

This database is freely accessible, and it is regularly updated having different ways to access the data and tools: Programmatic via APIs and R Bioconductor package, using a Web interface and via STRING download pages, where the complete interaction scores, as well as other information, are available [38, 39].

Statistical analysis

Analysis of variance (ANOVA), followed by Tukey's post hoc test, was applied to cell viability analysis. For analysis of gene expression was used *t* test. The data normality and variance homogeneity were previously tested. The results are expressed as mean ± S.E.M. Each experiment was performed three times using triplicates. In each experiment significance level was fixed at $P < 0.05$.

[1] Link for access STRING database: http://string-db.org/.

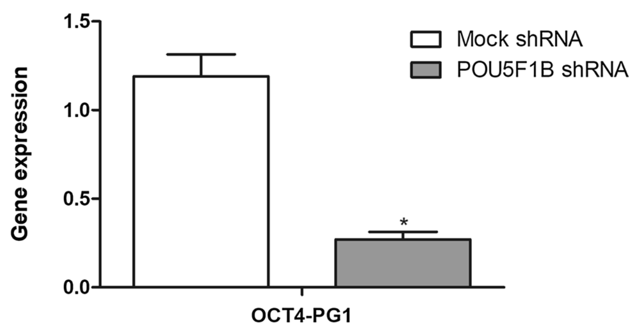

Fig. 1 Expression of *OCT4-PG1* pseudogene after genetic silencing. Comparison of expression of *OCT4-PG1* pseudogene between the POU5F1B shRNA (silenced cells) and Mock shRNA (control cells). Asterisk—means significant differences between mock and silenced cells ($P<0.05$). (Color figure online)

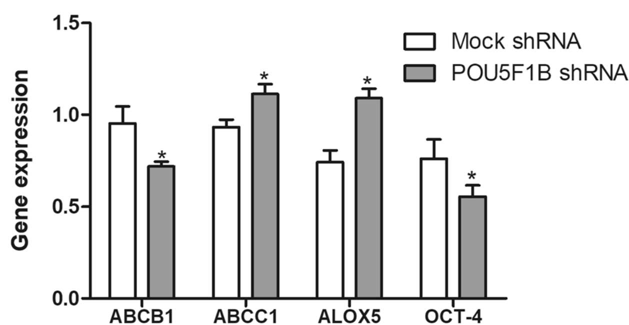

Fig. 2 Alteration of gene expression after silencing. Comparison of expression of *ABCB1, ABCC1, ALOX5, OCT-4* genes between the control (Mock shRNA) e silencing cells (POU5F1B shRNA). Asterisk—show significant differences between means of each gene ($P<0.05$). (Color figure online)

Results

Expression of OCT4-PG1 pseudogene

After silencing, the level of gene expression of the *OCT4-PG1* pseudogene was evaluated by real-time PCR. The results showed downregulation (4.4 fold) in the expression of the *OCT4-PG1* pseudogene in silenced cells compared with control cells (Fig. 1). Thus, we consider that gene silencing was effective.

Expression of genes

Considering that this work evaluates mechanisms related to the MDR phenotype, was analyzed the expression of the ABCB1 and ABCC1 genes after silencing. The expression of genes in FEPS cells were evaluated by real-time reverse-transcription PCR using specifics primers. We observed decreased expression levels of *ABCB1* gene and increased expression of the *ABCC1* gene in silenced cells compared with mock cells (Fig. 2). The results demonstrate alteration in the main genes related to the MDR phenotype. In addition, was evaluated the *OCT-4* gene which is regulated by the *OCT4-PG1* pseudogene and the *ALOX5* gene that regulates cell undifferentiation. In Fig. 2 we detected that the expression of the *OCT-4* gene was reduced in silenced cells compared with control cells. When *OCT-4* gene expression levels were decreased after silencing, the *ALOX5* gene expression levels in the cells were increased. These data represent a change in genes related to the characteristics of stem cells.

Expression of OCT-4 and ABCB1 protein

After alteration in gene expression levels, as seen in Fig. 2, was analyzed whether there would be alteration in the expression of ABCB1 and OCT-4 proteins after silencing by flow cytometry. For OCT-4 protein, the results showed that silenced cells (MFI = 17622.94) (Fig. 3b) expressed OCT-4 protein to a lesser extent than control cells (MFI = 34624.52) (Fig. 3a). The difference of median fluorescence intensity between cells labelled and cells unlabeled of Mock shRNA cells (MFI = 28406.43) and POU5F1B shRNA cells (MFI = 8665.82) (Fig. 3c) shows clearly the decreased of OCT-4 protein expression in silenced cells. Until now, the data show that the levels of OCT-4 gene and protein was reduced after silencing. Considering that the OCT-4 protein is a transcription factor that regulated genes expression related to MDR phenotype, was analyzed the ABCB1 protein expression. Similarly, the levels of ABCB1 protein expression were decreased in silenced cells compared to control cells (Fig. 4). The Table 2 with difference of median fluorescence intensity of cells labelled and cells unlabeled demonstrate the difference between ABCB1 protein expression of Mock shRNA (MFI = 91119.80) and POU5F1B shRNA cells (MFI = 18827.22 of peak 1). However, part of cells appears labeled for ABCB1 (MFI = 106713.21 of peak 2) although this peak contains only about 35% of the cells (Table 2). Thus, it is possible to suggest that the levels reduced of OCT-4 protein after silencing *OCT4-PG1* pseudogene, change the ABCB1 gene and protein expression related to MDR phenotype.

Activity of ABC transporters

In addition to the proteins expression, it is important to analyze if the ABCB1 and ABCC1 proteins extrusion activity were altered after silencing. The activity of ABCB1 was evaluated by using Rho 123 dye VP blocker and analyzed by fluorometry. As shown in Fig. 5a, mock cells presented

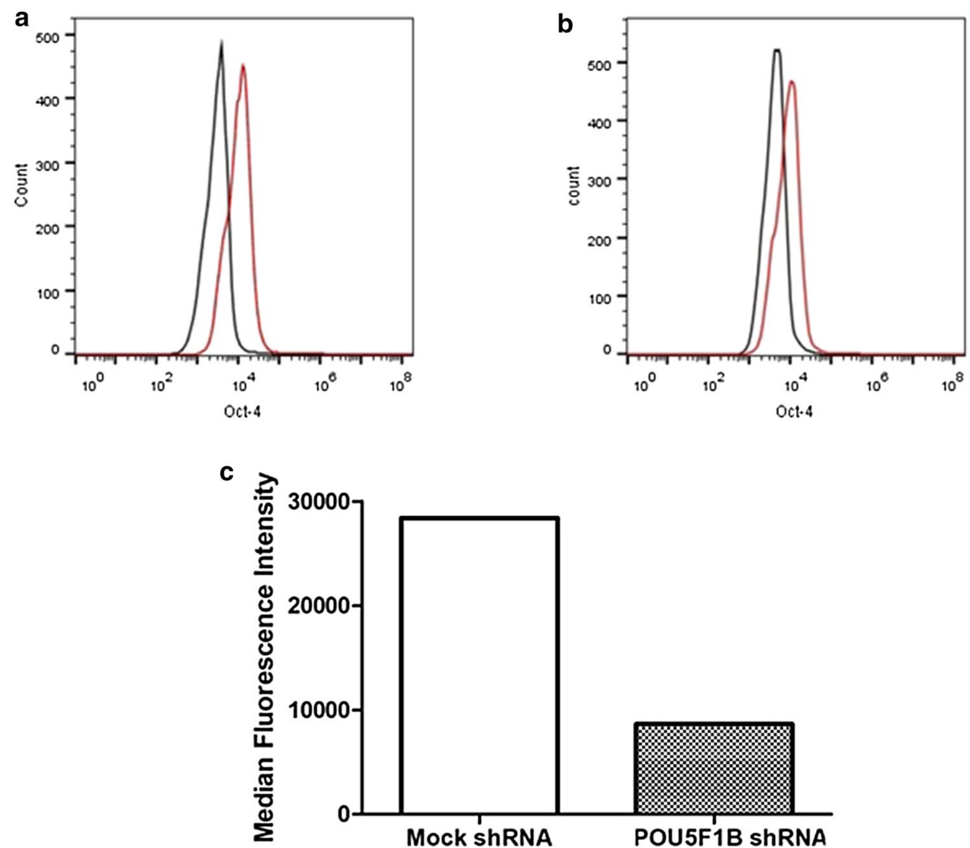

Fig. 3 *OCT4-PG1* silencing decreases OCT-4 protein levels. Representative histogram overlay of transfected FEPS cells with Mock shRNA (**a**) or POU5F1B shRNA (**b**), analyzing OCT-4 protein levels by flow cytometry. Difference of median fluorescence intensity of cells labelled and cells unlabeled of Mock shRNA and POU5F1B shRNA cells (**c**). Overlaid histograms show cells labelled with OCT-4 antibody (red line) versus unlabeled cells (black line). (Color figure online)

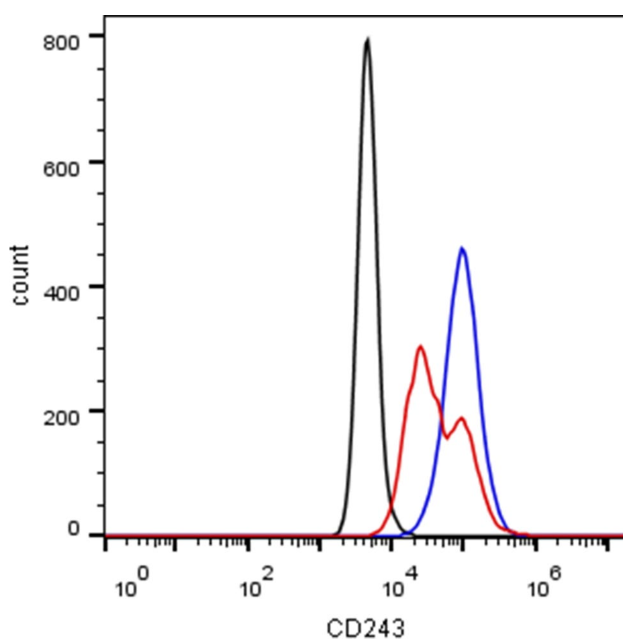

Fig. 4 *OCT4-PG1* silencing decreases ABCB1 protein levels. Representative histogram overlay of transfected FEPS cells with Mock shRNA or POU5F1B shRNA, analyzing ABCB1 protein levels by flow cytometry. Overlaid histograms show cells labelled with CD243 antibody for control cells (blue line), silenced cells (red line) and unlabeled cells (black line). (Color figure online)

Table 2 ABCB1 protein levels after *OCT4-PG1* silencing

ABCB1 protein expression	Mock shRNA	POU5F1B shRNA	
		Peak 1	Peak 2
MFI	91119.80	18827.22	106713.21
Percentage of cells	100%	65%	35%

Difference of median fluorescence intensity of Mock shRNA and POU5F1B shRNA cells labelled and cells unlabeled with CD243 antibody, and approximated percentage of cells at each peak of POU5F1B shRNA cells

lower fluorescence than silenced cells. When using VP blocker, the dye accumulated in mock cells. In silenced cells, the fluorescence was not altered in the presence of the blocker, suggesting that ABCB1 has low activity in this cell line. These results indicated that mock cells showed high activity of ABCB1 and the silenced cells has low activity of ABCB1. Thus, the results of ABCB1 activity in silenced cells corroborate with reduced of ABCB1 gene and protein expression.

In addition, the activity of the ABCC1 protein was evaluated in FEPS cells using CFDA dye, INDO blocker and analyzed by fluorometry. As observed in Fig. 5b, mock cells showed higher accumulation of fluorescent dye than silenced cells. Moreover, mock cells showed no

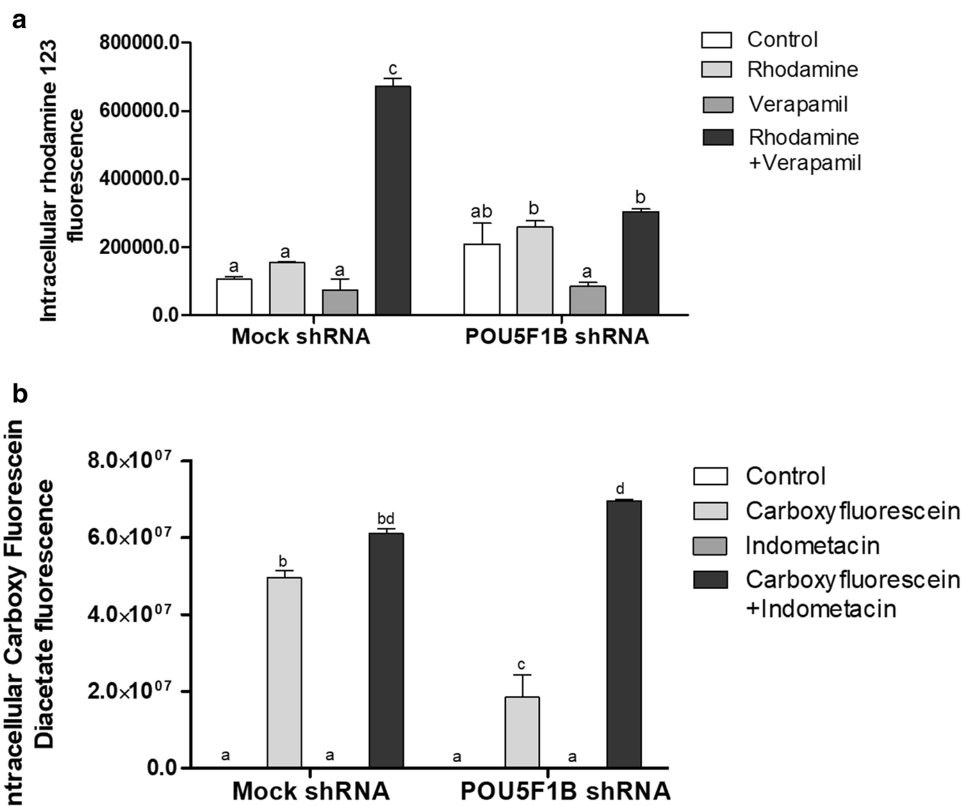

Fig. 5 Alteration in activity of ABCB1 and ABCC1 protein after *OCT4-PG1* silencing. Evaluation of ABCB1 activity by Rhodamine 123 dye extrusion (**a**) and of ABCC1 protein activity by CFDA fluorescence extrusion (**b**) in the mock and silenced cell line, by fluorimetry. The results are expressed as means ± S.E.M. Different letters indicate significant differences between the treatments ($P < 0.05$). (Color figure online)

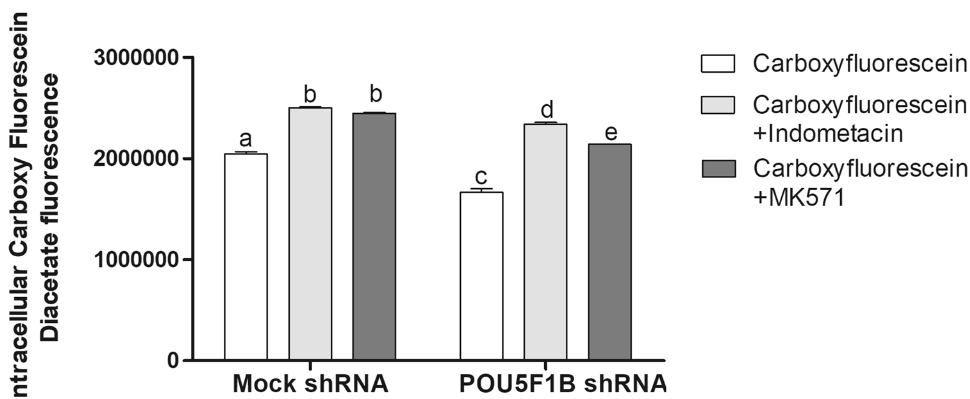

Fig. 6 Activity of ABCC1 protein in control and silenced cells. Evaluation of ABCC1 protein activity by CFDA fluorescence extrusion, INDO and MK571 blockers in the mock and silenced cell line, by flow cytometry. The results are expressed as means ± S.E.M. Different letters indicate significant differences between the treatments ($P < 0.05$). (Color figure online)

significant difference in fluorescence when the blocker INDO was added. However, the silenced cells presented higher fluorescence in the presence of the blocker than in its absence. These results suggest that the activity of ABCC1 was higher in silenced cells than in mock cells. The alteration of ABCC1 activity also can to observed when used the MK571 blocker. The results showed that silenced cells (MFI = 1.644.439) showed larger activity of the ABCC1 protein than control cells (MFI = 2.044.018) (Fig. 6). Taken together, all these results strongly demonstrate that the silencing of *OCT4-PG1* pseudogene altered the activity of proteins related to the MDR phenotype.

Sensitivity of cells to DNR or VCR

The FEPS cells have MDR phenotype, thus it becomes relevant to evaluate the sensitivity of this cell line after alteration in the activity of ABCs transporters. To determine the sensitivity of mock and silenced cells after *OCT4-PG1* pseudogene silencing, these cells were treated with the VCR or DNR chemotherapeutics. The cell growth was monitored for 72 h and number of viable cells was assessed by trypan blue. The results show that the silenced cells showed sensitivity to DNR alone and VCR alone, while mock cells did not show sensitivity to both chemotherapeutics (Fig. 7). From these

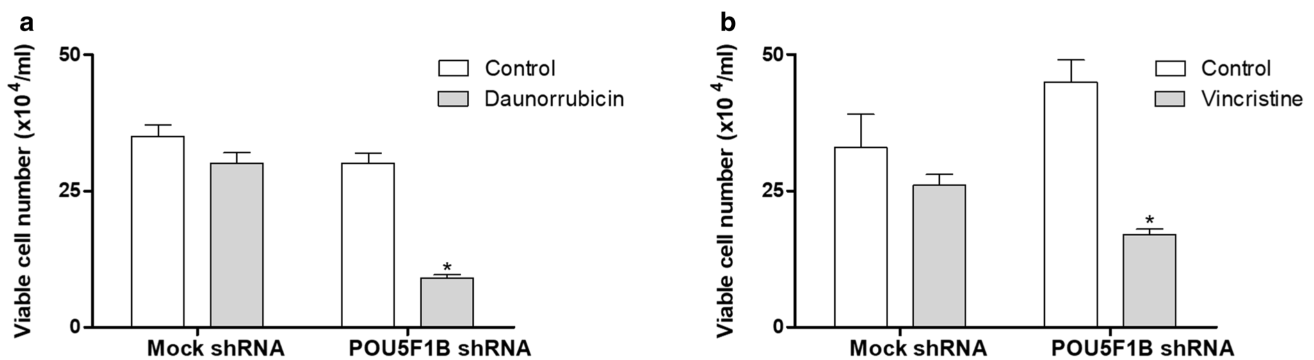

Fig. 7 Viable cell number after exposure to DNR and VCR. Analyses at 72 h of mock and silenced cells in the presence of 532 nM of DNR (**a**) and/or 60 nM of VCR (**b**) chemotherapeutics. Asterisk—means significant differences in relation to respective control. (Color figure online)

results we can observe a relation of the *OCT4-PG1* pseudogene with MDR phenotype.

STRING network

To better understand the relationship between the proteins examined in this work, a network structure was obtained from the STRING website. The names of four genes (OCT-4, OCT4-PG1, ABCB1, and ABCC1) were entered in the multiple gene search box. Then, the "minimum required interaction score" was set to "minimum" and the "more" option, which includes nodes that have interactions with the nodes informed by the user, was checked. With this option, the network depicted in Fig. 8 was obtained. This network was chosen to evaluate the possible interactions between OCT4-PG1 and OCT-4, ABCB1, and ABCC1. Note that the network presents edges with different colors representing distinct types of associations. Two groups of associations are represented: known interactions and other interactions. The network structure demonstrated that OCT4-PG1 (POU5F1B) interacts with OCT-4 through SOX2 and NANOG by experimental data, textmining and co-expression. The OCT-4 interacts with ABCB1 by textmining and co-expression, and with ABCC1, by textmining. For ABC transporters, the interaction with POU5F1B was indirect, possibly through its interaction with the network of transcripts formed between SOX2, NANOG and OCT-4 (Fig. 8).

Discussion

Phenotypic and functional heterogeneity in various types of cancer can be controlled by the self-renewal and differentiation ability of tumor-initiating cells [40]. The microenvironment of human embryonic stem cells (hESCs) has factors that may decrease the tumorigenicity of cancer cells by inducing apoptosis and decreasing cell proliferation [41]. Whereas the increase in proteins that are pluripotent stem cell markers may direct the reprogramming of malignant cancer cells [42]. According with these authors, human mammary carcinoma cells and human colorectal adenocarcinoma exposed to the microenvironment of hESCs increased the expression of OCT-4, SOX2, and NANOG proteins after internalization of exosome vesicles.

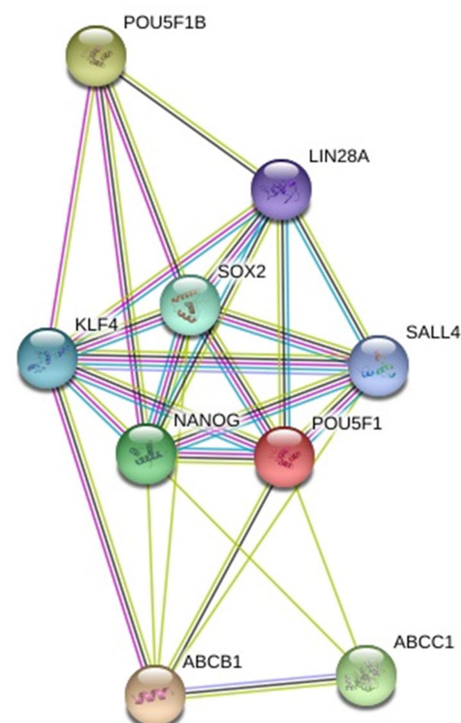

Fig. 8 Representation of network of the in vitro identified OCT4-PG1 pseudogene (POU5F1B) interactions. Known interactions the relationships are from curated databases (cyan) and experimentally determined (magenta). The other interactions presents associations obtained from textmining (green), co-expression (black) and protein homology (purple). Figure show simulations for the same group of genes with changes in the confidence score adopted: **a** highest confidence—0.900, **b** medium confidence—0.400, and **c** low confidence—0.150. (Color figure online)

OCT-4 has been shown to be involved in the CSCs properties of various cancers [43–46]. However, there are few data about the direction of mechanisms regulated by OCT-4 inside the cell [47]. Current scientific papers discuss the ability of pseudogenes to regulate the expression of the *OCT-4* gene in embryonic stem cells [44, 48, 49]. Scarola and coworkers (2015) [50] observed that the *OCT4-P4* pseudogene was responsible for induction and maintenance of *OCT-4* gene silencing in mouse embryonic stem cells.

In the present work, the *OCT4-PG1* pseudogene was silenced in the FEPS cell line. Considering that FEPS cells express high levels of the *OCT-4* gene [51], and the OCT-4 protein acts as a transcription factor for genes related to the MDR phenotype, it was relevant to evaluate whether a pseudogene can alter expression levels of the OCT-4 transcription factor in FEPS cell line. After silencing of *OCT4-PG1* pseudogene, the expression of OCT-4 protein and the *OCT-4* gene were reduced. These results indicate that the *OCT4-PG1* pseudogene might interfere in regulation of the expression of OCT-4 protein in the FEPS cell line. Therefore, it is possible to consider that the relationship between the gene and its pseudogene results in epigenetic gene regulation (as previously proposed by Scarola et al., 2015) [50]. Pseudogenes express miRNA that regulate their parental gene [33], thus OCT4-PG1 pseudogene may be regulating the expression of the OCT-4 through miRNA in the FEPS cell line.

Mechanisms of epigenetic regulation are directly related to the maintenance of SC characteristics. In cancer, the proliferation of CSCs can be altered after silencing of tumor suppressor genes induced by epigenetic changes. These factors contribute directly to the progression of several types of cancer [52]. The FEPS cell line expresses high levels of the *OCT-4* gene and low levels of *ALOX5* gene [51]. However, in this work the silenced cells showed expression levels of *OCT-4* and *ALOX5* altered. According to Chen et al. (2009) [53], in chronic myeloid leukemia cells, overexpression of the *ALOX5* gene is considered key to the regulation of leukemic stem cell function.

Carrett-Dias et al. [51] observed that the *ALOX5* and *ABCB1* genes presented an inverted expression profile among the K562, K562-Lucena1, and FEPS cell lines, showing higher expression of the *ALOX5* gene in K562 and lower expression in FEPS cells. Already, the *ABCB1* gene expression profile is reversed, such that K562 has lower expression and FEPS, higher expression. The K562-Lucena1 cell line presented intermediate expression between that of K562 and FEPS. When the K562 cell line was treated with an inducer of differentiation phorbol 12-myristate 13-acetate (PMA), the proportion was inverted. PMA treatment increased *ABCB1* and decreased *ALOX5* gene expression. Moreover, in the K562 and FEPS cell lines, the expression levels of the *ABCB1* and *OCT-4* genes vary. In FEPS cells, both genes show high expression compared with their K562 parental cell line, which is not resistant to multidrug [51].

It is relevant to recall that the promoter regions of the *ABCB1, ABCC1*, and *ABCG2* genes contain binding sites that interact with the transcription factor OCT-4 [16]. In this work, there was alteration in *ABCB1* and *ABCC1* genes expression levels which could be regulated by low levels of OCT-4 protein expression after silencing. The chemoresistance in CSCs occurs due extrusion of chemotherapeutic agents by the high activity of ABC transporters [52]. The ABCB1 and ABCC1 transporters are similar in their capacity to eject drugs from the intracellular medium. These proteins can transport a variety of hydrophobic drugs [20]; however, the ABCC1 protein shows specificity for organic anions and nonionic compounds, which may be transported as glutathione, glucuronide, or sulfate conjugates but may also be cotransported with glutathione [54, 55]. Here, when the level of *ABCB1* gene decreased in the silenced cells, the level of *ABCC1* gene increased, this event could represent a compensatory mechanism.

One of the strategies used to overcome the resistance acquired via ABC transporters is the use of inhibitors to block the function or expression of these proteins [56, 57]. Moreover, inhibition of ABC transporters can modify cellular processes such as migration, invasion, proliferation, and differentiation, thereby altering the tumor cell phenotype [58]. For example, indomethacin is a drug that acts as analgesic, anti-inflammatory, and antipyretic. This drug can inhibit MRP1 promoter activity [59], noncompetitively inhibit GST-Pi [60], non-selectively inhibit both cyclooxygenase (COX)-1 and COX-2 [61] and inhibit EGF-mediated calcium signals [62].

When the presence of ABCB1 protein was evaluated in this work it was possible to observe the existence of two populations of silenced cells, with the majority of cells had the ABCB1 protein expression reduced but some silenced cells maintained the high levels of ABCB1 protein (Fig. 4; Table 2). Since that the *ABCB1* gene depends on the degree of methylation of its promoter [63], changes in the promoter can decreasing the ABCB1 protein expression in some cells or maintain the expression similar to mock FEPS cells. This data is interesting because it shows that in a population of cells with MDR phenotype there may be more than one strategy to reach this phenotype and we should use more than one treatment strategy for attain the MDR phenotype.

Considering that both proteins ABCB1 and ABCC1 are directly related to the MDR phenotype of FEPS cells, it was relevant to evaluate the activity of these proteins following alteration of the genes encoding these efflux pumps. Therefore, an important aspect to evaluate is whether FEPS cell line resistance is altered after silencing. Considering that the silenced cells had lower ABCB1 activity, the results showing that the DNR remained in the intracellular medium, making

the cell sensitive to the chemotherapic, it was expected. A similar cytotoxic effect was observed when silenced cells were treated with DNR or VCR. In experiments on cellular viability, Daflon-Yunes and collaborators [35] observed that the FEPS cell line showed resistance to DNR and VCR and partial resistance to the chemotherapeutic IM (imatinib mesylate), while its K562 parental cell line showed sensitivity to all drugs. However, the higher sensitivity showed by the silenced cells when treated with DNR and VCR chemotherapic in this work confirm the role of ABCB1 protein in the multidrug resistance.

Multidrug resistance in the FEPS cell line may be associated with high levels of *OCT-4* gene expression. In a recent study, high expression levels of the *OCT-4* gene were observed in gefitinib-resistant lung cancer cells [46], which explains the sensitivity observed in our work.

The relationship between the genes examined in this work was evaluated by STRING website. In this result, it seems that the interaction of POU5F1B with POU5F1 occurs indirectly through the network of transcripts formed between OCT-4, SOX2, and NANOG. These transcriptional factors form a circuit that controls the pluripotency of embryonic stem cells [64]. It is important to observe that the OCT-4 interacts directly with ABCB1 and with ABCC1. These data were expected because, as previously mentioned, the OCT-4 is a transcription factor that interacts with the binding site of the ABC family [16]. For ABC transporters, the interaction with POU5F1B was indirect, possibly through its interaction with the OCT-4.

Conclusion

The present work has demonstrated that silencing of the pseudogene *OCT4-PG1* caused a decrease in OCT-4 gene and protein expression, and this effect altered ABC transporters. The expression and activity of *ABCB1* were reduced. Although the gene expression and activity of ABCC1 had been increased maybe as a compensatory mechanism, the cell sensitivity to DNR and VCR chemotherapics was increased. Moreover, the *ALOX5* gene which controls cell undifferentiation, was more expressed when some characteristics of the MDR phenotype were decreased. It was also possible to demonstrate with a network that the OCT4-PG1 interacts with the OCT4, which interacts directly with ABCB1 and ABCC1. Based on these results, it is possible to propose that OCT4-PG1 presented in CSCs can influence multidrug resistance in the FEPS cell line.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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CAPÍTULO II

ACQUISITION OF THE MULTIDRUG RESISTANCE PHENOTYPE CHANGES GENES RELATED TO STEM CELLS CHARACTERISTICS IN CHRONIC MYELOID LEUKEMIA

(a ser submetido para a Revista *Cancer Letters*)

Acquisition of the multidrug resistance phenotype changes genes related to stem cell characteristics in chronic myeloid leukemia

Aline Portantiolo Lettnin [a,b], Eduardo Felipe Wagner [b], Andrés Delgado Cañedo[c], Vivian Mary Rumjanek [d], Gilma Santos Trindade [a], Ana Paula de Souza Votto [a,b*]

[a] Post-Graduate Program in Physiological Sciences - PPGCF, Federal University of Rio Grande - FURG, Avenue Italia, Km 8, Zip code 96203-900, Rio Grande, RS, Brazil.

[b] Laboratory of Cell Culture, Institute of Biological Sciences - ICB, Federal University of Rio Grande - FURG, Avenue Italia, Km 8, Zip code 96203-900, Rio Grande, RS, Brazil.

[c] Federal University of Pampa - UNIPAMPA, Avenue Antônio Trilha, 1847, Zip code 97300-000, São Gabriel, RS, Brazil.

[d] Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, Avenue Carlos Chagas Filho, Zip code 21941-902, Rio de Janeiro, RJ, Brazil.

*Corresponding author e-mail: anavotto@yahoo.com.br (Ana P. S. Votto)

Institutional e-mail address: anavotto@furg.br

Instituto de Ciências Biológicas - Universidade Federal do Rio Grande – FURG

Avenida Itália, Km 8, s/n, Zip code 96203-900 Rio Grande/RS – Brazil

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CSC: cancer stem cells; DNR: daunorubicin; hESC: human embryonic stem cells; MDR: multidrug resistance; MRP1: multidrug resistance associated protein; P-gp: P-glycoprotein; SC: stem cells; VCR: vincristine; 5-LO: 5-lipoxygenase.

Abstract

The present work aimed to evaluate genes related to stem cells (SC) characteristics during the acquisition of the multidrug resistance (MDR) phenotype in the K562 cell line of chronic myeloid leukemia (CML). The cell sensitivity to the chemotherapeutic agents vincristine (VCR) and daunorubicin (DNR), gene expression level, ABC transporters and the presence of its encoded protein at plasma membrane were analyzed. Results showed that K562 cells treated with VCR displayed an increase the expression *ABCB1* gene expression and a decrease of *ALOX5* gene expression. Same results were achieved when K562 cells were treated with DNR. Using MDR cell lines K562-Lucena and FEPS, silencing *ABCB1* in both cell lines led to an increased expression of *ALOX5* gene compared to control cells. The results presented in this work suggest that the acquisition of MDR phenotype could further decrease *ALOX5* gene expression. So, this pathway could be considered for the development of new strategies to increase the sensitivity of leukemic cells that had acquired MDR phenotype.

Keyword: *ALOX5* gene; Cancer stem cells; Daunorubicin chemotherapeutic; K562 cell line; Vincristine chemotherapeutic.

1. Introduction

Leukemia comprises a diverse group of diseases characterized by the clonal proliferation of blood progenitor neoplastic cells (Guo et al., 2014). The chronic myeloid leukemia (CML) is a type of leukemia formed by malignant clonal hematopoietic stem cell (HSC) with distinct stages of differentiation (Gerber et al., 2011) and begins with the fusion of *BCR* (breakpoint cluster region) and *ABL* (Abelson Murine Leukemia) genes, which create a constitutively active BCR-ABL tyrosine kinase protein (Chereda & Melo, 2015).

Some cancers are composed by a heterogeneity of cancer stem cells (CSC) that gave rise to the tumor. In this situation, it is possible that the tumor growth and cancer progression may be regulated by CSC, possibly due to resistance to therapy (Oravecz-Wilson et al., 2009). Considering that the heterogeneity can keep the tumoral resistance, this factor is relevant for the development of effective therapies against cancer (Dagogo-Jack & Shaw, 2017).

In CML there is a group of leukemia initiating cells with capacity of self-renewal and cellular differentiation, characteristic of stem cells (SC) (Graham et al., 2007). These cells also show the period of cell division similar to differentiated leukemia stem cells (Graham et al., 2007). These properties suggest that the initial leukemia cells can be SC.

The *ALOX5* gene can act regulating some SC characteristics in leukemia. At the same time, the deficiency or inhibition of *ALOX5* gene may prevent the leukemic process induced by BCR-ABL oncoprotein, and, on the other side, the BCR-ABL protein can upregulate the expression of the *ALOX5* gene (Chen et al., 2009). The inhibition of *ALOX-5* gene expression may direct rapid clinical diagnosis to detect acute myeloid leukemia (Roos et al., 2014).

The *ALOX5* gene is primarily expressed in peripheral blood monocytes, polymorphonuclear leukocytes, macrophages and mast cell (Woods et al., 1995). In leukemia, the overexpression of *ALOX5* gene was verified in CML stem cells in a murine leukemia and also in human CML cells (Chen et al., 2009).

The *ALOX5* gene encode the 5-lipoxygenase (5-LO) enzyme that is activated by 5-lipoxygenase-activating protein (FLAP), a nuclear membrane-bound protein. This enzyme is important on the conversion of arachidonic acid for leukotriene A4 (LTA4) biosynthesis (Woods et al., 1995), which is considered a biologically active lipid mediator participating in malignant diseases and inflammatory processes (Poczobutt et al., 2016).

The enzymes involved in prostaglandins and leukotrienes synthesis are expressed during cancer-related inflammation and influence the inflammatory responses (Greenhough et al., 2009). The resultant leukotrienes of the binding of arachidonic acid by 5-LO enzyme, make the leak of vascular endothelium and this process contribute for cancer metastasis (Gupta et al., 2007).

Carrett-Dias and collaborators (2016) showed that *ALOX5* gene is overexpressed in the K562 cell line, which was derived from a patient diagnosed with CML, but its expression is reduced in K562-Lucena and FEPS cell lines, cells that present an MDR phenotype. Moreira et al. (2014) also observed a decrease in ALOX5 expression in the FEPS cells in relation to K562 cells. Moreover, the *ABCB1* gene expression presents an inverse profile to that observed for *ALOX5* gene in the studied cell lines (Carrett-Dias et al., 2016). The K562-Lucena and FEPS were originated from K562 cells. The K562-Lucena cell line was developed by increasing concentrations of the chemotherapeutic drug vincristine (VCR) until acquisition of MDR phenotype with 60 nM of VCR (Rumjanek et al., 1994 and 2001). This chemotherapeutic agent destabilizes the cytoskeleton of the cell, acting as a blocker for microtubule polymerization (Himes et al.,

1976). The FEPS cell line was developed by increasing concentrations of the chemotherapeutic drug daunorubicin (DNR) until acquisition of MDR phenotype with 532 nM of DNR (Daflon-Yunes et al., 2013). This chemotherapeutic agent inhibits the action of the enzyme Topoisomerase II in the DNA inducing cell death (Nielsen et al., 1996).

In cancer, the high expression of the ABC transporter family is one of the factors that establish the acquisition of MDR phenotype (Gottesman et al., 2002). The ABC transporters family have different proteins already characterized, however, for MDR phenotype the major ABC proteins investigated as regulators are MDR1 (P-glycoprotein [P-gp], ABCB1), MRP1 (multidrug resistance-associated protein 1, ABCC1) (Gottesman et al., 2002; Wu et al., 2011).

ABC transporters confer resistance to chemotherapeutic agents by the ability to transport a broad range of hydrophobic drugs in different cancer types (Lu et al., 2017). However, the ABC transporters may play other important roles in cancer biology (Fletcher et al., 2010) as alteration in cell cycle and inhibition of apoptosis (Katoh et al., 2008). The members of ABC family can transport different classes of drugs such as amphipathic anions, non-ionic lipophilic compounds, hydrophobic drugs, amphipathic drugs conjugated with glutathione or sulphates (Loe et al., 1998; Mohammad et al., 2018). This ability to eject drugs to the extracellular medium renders the cells resistant to various chemotherapeutic agents. Currently, it is known that in the tumor microenvironment cancer cells share microvesicles that transfer MDR proteins for cancer cells no-MDR and reshape the immune system. This allows for an escape of the immune system, survival and progression of cancer (Jaiswal et al., 2017). In SC, it is possible to find high levels of expression of specific ABC transporters, which are directly related to the multidrug resistance (MDR) phenotype (Dean et al., 2005).

Marques and coworkers (2010) observed in the K562 and K562-Lucena cell lines that the promoter regions of the *ABCB1*, *ABCC1* and *ABCG2* genes contain binding sites that interact with the transcription factor OCT-4, which is considered a marker of CSCs, besides verifying an overexpression of *OCT-4* gene in the MDR K562-Lucena cells. This was also later observed by Carrett and collaborators (2016) also in the MDR FEPS cells.

This ability of leukemia SC to resist treatment with chemotherapeutic agents may be related to the acquisition of the MDR phenotype. Therefore, the present work aimed to evaluate genes related to SC characteristics during the acquisition of the MDR phenotype, as a means to understand some of the mechanisms of resistance to multiple drugs in the CML cell line K562.

2. Materials and methods

2.1. Cell and Culture Condition

The K562, K562-Lucena and FEPS cell lines were obtained from the Tumoral Immunology Laboratory at the Medical Biochemistry Institute of the Federal University of Rio de Janeiro (Brazil). The cells were grown in medium RPMI1640 (Gibco, USA) supplemented with sodium bicarbonate (2.0 g/L) (Vetec, Brazil), L-glutamine (0.3 g/L) (Vetec, Brazil), 10% fetal bovine serum (Gibco, Brazil), 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 μg/mL] and amphotericin B [0.25 μg/mL]) (Gibco, USA). In order to preserve the MDR phenotype, the K562-Lucena cell line was maintained with 60 nM of vincristine (VCR) and the FEPS cell line with 532 nM of daunorubicin (DNR) chemotherapeutics. The cells received the chemotherapeutics every three days, respecting the cell cycle.

The K562-Lucena and FEPS cell lines denominated Mock shRNA (control) and ABCB1 shRNA (silenced) were also obtained from the Tumoral Immunology Laboratory

at the Medical Biochemistry Institute of the Federal University of Rio de Janeiro (Brazil). These cells were silenced for the ABCB1 by transfection with shRNA plasmids provided in the Qiagen SureSilencing kit (QIAGENTM, Dusseldorf, Germany). The ABCB1 shRNA plasmid used was the KH01527N (Daflon-Yunes et al., 2013). The cells received the plasmid for silencing and a control plasmid for comparison of the results, thus obtaining a control cell and a silenced cell. Transfected K562-Lucena and FEPS cells were maintained at a concentration of 2×10^4 cells/mL in 24 well culture plates at 37 ºC in a 5% CO_2 humidified environment. For maintenance of successfully transfected cells it was added G418 geneticin (Sigma Chemical Co™., St. Louis, USA) in the concentration 0.5 mg/mL.

2.2. Cell viability

K562 cell line was centrifuged, suspended in RPMI 1640 medium and maintained in 96-well culture plates to a final concentration of 2×10^4 cells/mL. To evaluate the sensitivity, the cells were treated with the concentrations 3.75; 7.5; 15; 30; 60 nM of VCR chemotherapeutic, and 4.15; 8.31; 16.63; 33.25; 66.5; 133; 266 and 532 nM of DNR chemotherapeutic. The percentage of cell viability were assessed by trypan blue (Gibco, USA) exclusion assay in 0, 24, 48, 72 and 96 h after incubation of K562 cells with VCR or DNR.

2.3. Resistance induction in K562 cells with the chemotherapeutic drugs VCR or DNR

K562 cells were maintained in 24-well culture plates to a final concentration of 2×10^4 cells/mL. The cells were exposed to crescent concentrations of VCR (3.75; 7.5; 15; 30 and 60 nM), and DNR (4.15; 8.31; 16.63; 33.25; 66.5; 133; 266 and 532 nM).

K562 cells were initially exposed at lower the concentration of VCR (3.75 nM) or DNR (4.15 nM) and were maintained with these concentrations until reaching 100% cell viability. This was followed by the cells receiving the next concentrations of the chemotherapeutic agent until the highest concentrations of each chemotherapeutic (60 nM of VCR and 532 nM of DNR) was reached. At the moment that the cells reach 100 % of viability at each concentration (5 VCR concentrations and 8 DNR concentrations) RNA extraction was performed.

2.4. Gene expression analysis

2.4.1. Total RNA extraction and cDNA synthesis

Total RNA was extracted from six samples of each cell line with an amount of 2×10^4 per sample cells according to the protocol of the manufacturer of TRIzol Reagent (Invitrogen, Brazil). The quality and quantity of RNA was measured by BioDropµLite spectrophotometer (BioDrop, England). The RNA integrity was confirmed by electrophoresis in 1.5% agarose gel with ethidium bromide (0.5µg/mL). cDNA synthesis was performed by reverse transcription of 1 µg RNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Brazil).

The K562 cell line was maintained in 24-well culture plates at 2×10^4 cells/mL. Total RNA was extracted from cultures that were 100% alive after each chemotherapeutic concentration was reached: 3.75; 7.5; 15; 30 and 60nM VCR, and 4.15; 8.31; 16.63; 33.25; 66.5; 133; 266 and 532nM for DNR. Total RNA was also extracted from K562-Lucena and FEPS cells silenced for *ABCB1* gene for gene expression.

2.4.2. Gene expression

The gene expression analysis was performed by qRT-PCR using a Real-time PCR System 7300 equipment (Applied Biosystems, Brazil). PCR reactions were carried out

using Gotaq PCR Master Mix (Promega Corporation, Brazil). Gene-specific primers (Table 1) were designed based on sequences available in GenBank using the Primer Blast tool (http://www.ncbi.nlm.nih.gov). Previously, the PCR amplification efficiency of each primers pair was evaluated by serial dilutions reactions where the efficiency of reactions showed appropriate parameters (Table 1). The *EF1α* and *B2M* genes were chosen as reference genes after presented stability when tested with geNorm applet (Vandesompele et al., 2002). Normalization factor was calculated as the geometric mean of the expression values of the reference genes tested by geNorm applet. Relative expression levels of the target genes are calculated by values of the standard curve.

2.5. Analysis of protein expression by flow cytometry

In samples of K562, K562-Lucena, FEPS cells we analyzed the ABCB1 protein expression at plasma membrane by flow cytometry. Similarly, this was done with K562 cells treated with 3.75 or 60 nM of VCR and K562 cells treated with 4.15 or 532 nM of DNR. To measure ABCB1 protein expression at the plasma membrane by flow cytometry 10^5 cells were spun down at 1000 xG during 5 minutes and resuspended in 20 µl of complete culture medium; to this suspension 2 µl of anti-ABCB1 antibody was added. The antibody used was FITC mouse anti-human ABCB1 clone 17F9 antibodies (Becton, Dickinson and CompanyTM, Franklin Lakes, USA). Samples were incubated during 30 minutes in the dark. This was followed by the addition of 280 µl of complete culture medium and each sample was analyzed adquiring 10000 event gated live cells and evaluating the florescence in FL1 filter.

2.6. Statistical analysis

Analysis of variance (ANOVA), followed by Tukey's post hoc test, was applied to all analyses, except for some gene expression analyses, when the Student's *t*-test was

used. The data normality and variance homogeneity were previously tested. The results are expressed as mean ± S.E.M. Each independent experiment was repeated three times with a minimum of triplicate samples. In each experiment significance level was fixed at $p<0.05$.

3. Results

3.1 Sensitivity of K562 cells to VCR

K562 cells do not have a MDR phenotype, thus it becomes relevant to evaluate the sensitivity of this cell line after exposure to the chemotherapeutic drugs VCR or DNR. The results show that 15; 30 and 60 nM concentrations tested induced a decrease in cell viability at 48 and 72 h after treatment. At 96h all concentrations tested induced a decrease in cell viability (Fig 1).

3.2 Sensitivity of K562 cells to DNR

The sensitivity of the K562 cell line was also analyzed with 4.15; 8.31; 16.63; 33.25; 66.5; 133; 266 and 532 nM concentrations of the chemotherapeutic DNR. These experiments were done in order to demonstrate the sensitivity of this cell line to chemotherapeutic agents. K562 cells treated with concentrations above 33.25 nM decreased cell viability at 96 h (Fig 2).

After assessing sensitivity to chemotherapeutic agents, K562 cells were maintained in a 24-well plate by adding increasing concentrations of VCR and DNR.

3.3 Gene expression of K562 exposed to VCR

In this work, we evaluated the relationship genes expression related to stem cells in K562 cell line with the acquisition of the MDR phenotype. Thus, after analyzing the

sensitivity of the cell line to the chemotherapeutic VCR or DNR, gene expression levels were evaluated with each increasing concentration of the chemotherapeutic added.

K562 cells were exposed to the concentrations 3.75; 7.5; 15; 30; 60 nM of VCR and *ABCB1*, *ABCC1* and *ALOX5* genes were evaluated.

K562 cells treated with VCR showed an increase in the levels of *ABCB1* gene expression from the concentration of 7.5 nM VCR. For *ALOX5* gene, the levels of expression decrease when 3.75; 15; 30 and 60 nM of VCR was used. However, the *ABCC1* gene expression was not changed (Fig 3). These results indicate that in the process of acquisition of the MDR phenotype, the expression levels of the *ABCB1* gene increase even with the lower concentrations of VCR, whereas, only in the 15 nM concentration there is a reduction in expression levels of the *ALOX5* gene.

3.4 Gene expression of K562 exposed to DNR

The gene expression profile of the K562 cell line was also analyzed with 4.15; 8.31; 16.63; 33.25; 66.5; 133; 266 and 532 nM of DNR.

Similarly to results observed after exposure to VCR, K562 cells treated with different concentrations of DNR had an increased expression of the *ABCB1* gene from the concentration of 66.5 nM DNR onwards. For *ALOX5* gene, the levels of expression decreased from the concentration of 16.6 nM DNR. However, no change in the *ABCC1* gene expression was observed (Fig 4). These results showed that expression levels of the *ALOX5* gene were reduced at a lower DNR concentration than that in which the expression levels of the *ABCB1* gene were increased.

3.5 ABCB1 protein expression

After alteration in gene expression levels, as seen in Fig 3 and 4, it was analyzed whether there would be alteration in the expression of ABCB1 protein. This analyze is

important for demonstrated the basal levels of ABCB1 protein in the K562, K562-Lucenaande FEPS cells and compare with transformed K562 cells in this work.

In the K562 cells, the results showed low protein expression between the labeled and unlabeled cells (MFI = -335.74) (Fig 5 A). For K562 cells exposed to 3.75 nM concentration of VCR the ABCB1 protein expression was low (MFI = -259.49) (Fig 5 D) compared to K562 cells exposed to 60nM concentration of VCR (MFI = 57821.71) (Fig 5 E). These data are in acordance with that seen in K562-Lucena cells (which were selected with VCR) that demonstrated an increased expression of the protein in labeled cells compared to unlabeled cells (MFI = 46100.930) (Fig 5 B).

For K562 cells exposed to 4.15 nM concentration of DNR the ABCB1 protein expression was low (MFI = 478.35) (Fig 5 F) compared to K562 cells exposed to 532 nM concentration of DNR (MFI = 30955.66) (Fig 5 G). The ABCB1 protein expression on FEPS cells (which were selected with DNR) was the highest (MFI = 3603716.483) (Fig 5 C).

3.6 Gene expression of ABCB1 silenced K562-Lucena cells

In order to evaluate the opposite, i.e. the "loss" of the MDR phenotype, it was analyzed the *ABCB1*, *ABCC1*, *ALOX5* and *OCT-4* genes expression in K562-Lucena and FEPS cell lines silenced for *ABCB1*. However, it is important to take into account that the silencing process of the *ABCB1* gene may have altered other features of the MDR phenotype.

The results demonstrate that K562-Lucena cells silenced for *ABCB1* gene showed a decrease in *ABCB1* gene expression, as expected, proving the gene silencing. However, a significant increase in *ABCC1* gene expression was observed, in silenced cells compared to control cells (Fig 6).

Looking for stem cells characteristics, the expression of *ALOX5* and *OCT-4* genes was analyzed. No significant differences in the *OCT-4* gene expression was observed. However, *ALOX5* gene showed an increased expression in silenced cells compared to control cells (Fig 6).

3.7 Gene expression of ABCB1 silenced FEPS cells

As previously mentioned, the FEPS cell lines was silenced for the *ABCB1* gene. Thus, the gene expression of *ABCB1*, *ABCC1*, *ALOX5* and *OCT-4* was analyzed in this cell.

Similar to the results observed in transfected K562-Lucena cells, the *ABCB1* gene expression was decreased in FEPS cells silenced for *ABCB1* gene that proves the gene silencing (Fig 7). Considering that silenced FEPS cells demonstrated a decrease in a gene related to MDR phenotype, we analyzed the expression of *ALOX5* and *OCT-4* genes expression for investigating stem cells characteristics.

The *OCT-4* gene expression was decreased, but *ALOX5* gene showed increased expression in silenced cells compared to control cells (Fig 7).

4. Discussion

Cancer biology has been attempting to better understand the drug resistance mechanisms acquired by tumor cells during disease treatment. Despite intensive research curative therapy of various cancers has been limited due to lack of knowledge related to the physiological and molecular mechanisms that regulate cancer progression, recurrence and metastasis (Kavalerchik et al., 2008; Holohan et al., 2013). In this work, we seek an improved understanding of the relationship between MDR phenotype and genes related to SC characteristics from molecular mechanisms in different cell lines of CML.

In myeloid leukemia treatment tyrosine kinase inhibitors (TKIs) are used for the control of the chronic phase of the disease, however, cancer can remain due to the presence of leukemia SC (Kavalerchik et al., 2008). The inhibition occurs by binding of inhibitor in the BCR-ABL kinase domain, thereby preventing the enzymatic activity of the protein and its oncogenic signaling pathways (Goldman & Melo, 2003). However, the addition of the chemotherapeutic drug stimulates the overexpression of *ABCB1* gene, which may support the activation of other mechanisms of resistance beyond the extrusion of the chemotherapeutic agent (Eadie et al., 2016). Advanced research based on molecular or cytotoxic pathways are aimed to eliminate cancer, nevertheless the growth and progression of cancer can be regulated by CSC which are not studied efficiently (Roos et al., 2014).

The CSC are a population of cells characterized by self-renewal capacity and great carcinogenic potential. These characteristics make it possible to tumor recurrence due to an enhanced chemoresistance (Beck et al., 2013). Therefore, it is possible that CSC would be highly resistant to conventional chemotherapies because of some characteristics as high expression of ABC transporter proteins (Dean, 2009).

In the present work, K562 cells were exposed to various concentrations of the chemotherapeutic drugs VCR and DNR. These same drugs were subsequently used for the induction of resistance. The result indicates that the sensitivity of the K562 cell line to VCR started at 3.75 nM. This concentration is 16-fold lower than 60 nM (concentration that selects cell resistance to VCR). For DNR, the sensitivity of the K562 cell line started at 33.25 nM. This concentration is 16-fold lower than 532 nM (concentration that selects cell resistance to DNR)

Subsequently, cells were exposed to increasing concentrations of VCR or DNR and the analysis of gene expression was performed to evaluate the possible relation

between the acquisition of resistance to chemotherapeutic and the expression of some SC characteristics. We were able to show that K562 cells the overexpression of the *ABCB1* gene started at the concentration of 7.5 nM VCR. This concentration is 8-fold lower than 60 nM (concentration that selects cell resistance to VCR). The *ALOX5* gene was overexpressed at 15 nM of VCR and this concentration is 4-fold lower than 60 nM. These data show that K562 cells activate the molecular mechanisms for chemotherapeutic resistance at lower concentrations of VCR and express SC characteristics at intermediate concentrations of VCR.

Similar results were observed by Gromicho and collaborators (2011), they demonstrated that K562 cells exposed to different chemotherapeutic overexpress *ABCB1* gene with either 1.5 nM dasatinib, or 1.0 μM of imatinib. According to the authors, the increases in ABCB1 transporter expression can be relevant in the early stages of resistance acquisition. In another work, K562 cells overexpressed the ABCB1 protein in 2 μM VCR (Souza et al., 2011). The authors suggest that this protein can be regulated by VCR treatment and may play an anti-apoptotic role in K562 cells.

Considering that the VCR chemotherapeutic destabilizes the cell cytoskeleton l, acting as a blocker of the microtubule polymerization (Himes et al., 1976; Owellen et al., 1977), it is possible that the cell could activates the *ABCB1* gene transcription for extrusion of the chemotherapeutic already in lower concentrations of VCR.

When K562 cells were exposed to DNR chemotherapeutic, the *ALOX5* gene was upregulated at 16.6 nM of DNR, this concentration is 32-fold lower than 532 nM. Already the *ABCB1* gene was overexpressed with 66 nM of DNR, and this concentration is 8-fold lower than 532 nM. These data suggest that K562 cells actived the molecular mechanisms for SC pathway expression at lower DNR concentrations and expressed mechanisms for resistance to DNR at intermediate concentrations. The cancer cells after the malign

transformation can start to express stem characteristics (Martinez-Climent et al., 2006), which could collaborate in the acquisition of MDR phenotype.

As expected, the lowest chemotherapeutic concentrations used for the treatment of the K562 cells (3.75 nM VCR and 4.15 nM DNR) were not able to induce the expression of the ABCB1 protein. However, at the two highest concentrations, 60 nM VCR and 532 nM DNR, the K562 cells expressed high levels of the ABCB1 protein. Thus, it is possible to suggest that the increased levels of ABCB1 protein in the higher concentrations of VCR or DNR can induces the acquisition of MDR phenotype in the transformed K562 cells transformed.

In this work, we demonstrate transformed K562 cells had possibly acquired the drug resistance, considering that both the ABCB1 gene and protein expression were elevated after exposure to VCR or DNR, whereas the acquisition of MDR phenotype can occur by the expression of ABCB1 protein induced by chemotherapy (Thomas & Coley, 2003). Moreover, K562 cells treated with VCR and DNR reduced the *ALOX5* gene expression while acquiring the MDR phenotype. The chemotherapeutic agent VCR was able to cause downregulation of the *ALOX5* gene expression (99.28 %), while the *ALOX5* gene was downregulated in 70,30 % during DNR treatment.

We have previously demonstrated that FEPS cells silenced for *OCT4-PG1* presented alterations in gene expression related to the MDR phenotype. After silencing, the cells showed low expression of *ABCB1* and *OCT-4* genes, while the *ALOX5* and *ABCC1* genes were upregulated (Lettnin et al., 2019). This cellular behavior suggests that alteration in the mechanism related to the MDR phenotype modified the gene expression profile related to stem characteristics, similar to the results observed in this work.

As a way of comparing the data, we analyzed *OCT-4* and *ALOX5* gene expression in K562-Lucena and FEPS cells silenced *ABCB1*. Although *OCT-4* gene expression there

was not altered in K562-Lucena cells silenced *ABCB1*, this gene was reduced in FEPS cells silenced. This result can contribute for alteration MDR phenotype once that OCT-4 protein contains binding sites that interact with promoter regions of the *ABCB1*, *ABCC1* and *ABCG2* genes (Marques et al., 2010). Moreover, high levels of expression of specific ABC transporters can find in SC (Dean, 2005). For *ALOX5* gene we found significant overexpression . The activation of the *ALOX5* gene expression can induce characteristic and profile of CSC and it is necessary to consider that K562, K562-Lucena and FEPS cells shows differences in the *ALOX5* gene expression (Carrett-Dias et al., 2016). As observed by our group, the *ALOX5* gene is overexpressed in the K562 (not MDR), low expression is observed in the FEPS (MDR) and an intermediate expression is observed K562-Lucena (MDR), when compared to K562 and FEPS cell lines. These data were confirmed in this work, observing increased *ABCB1* gene expression and decreased *ALOX5* gene expression in K562 cells exposed to the highest concentrations of VCR and DNR chemotherapeutics. The opposite was demonstrated when K562-Lucena and FEPS cells silenced for *ABCB1*; in this case, a significant increase in *ALOX5* gene expression was shown, similar to the profile of non-resistant K562 cells. These data may suggest that there is an inverse relationship between the expression of *ALOX5* gene and the acquisition the MDR phenotype.

5. Conclusion

The results presented in this work suggest that the acquisition of MDR phenotype by cancer cells could be associated to the decrease of *ALOX5* gene expression, that is very important for CML functions. Therefore, this pathway could be considered for development of new strategies to sensitize leukemic cells that acquired MDR phenotype. Hence, new studies on the molecular mechanisms of leukemic should be encouraged.

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7. Compliance with ethical standards

7.1. Conflicts of interest: The authors declare that they have no conflict of interest.

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7.3. Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1 - Primers sequences used in gene expression analysis

Gene	Primers sequence 5’ – 3’	Primers Efficiency(%)	GenBank accession number
ALOX5	F: GTGGCGCGGTGGATTC	94,99	XM_011539564
	R: TGGATCTCGCCCAGTTCCT		
ABCB1	F:CCTCAGTCAAGTTCAGAGTCTTCA	102,01	NM_000927
	R:CTCCACTTGATGATGTCTCTCACT		
ABCC1	F: GGATCTCTCCAGCCGAAGTCT	99,25	XM_017023237.1
	R: GTGATGGGAGCCAGAAGCA		
OCT-4	F: TTCCCCATGGCGGGACACC	96,09	NM_002701
	R: CCCCTGGCCCATCACCTCC		
EF1α	F: GCCAGTGGAACCACGCTGCT	103,14	NM_001402
	R: ATCCTGGAGAGGCAGGCGCA		
B2M	F: CTCACGTCATCCAGCAGAGAA	98,19	NM_004048.2
	R: TCGGATGGATGAAACCCAGAC		

OCT4: POU class 5 homeobox 1 (POU5F1) transcription factor; *ABCB1*: ATP-binding cassette, sub-family B (MDR); *ABCC1*: ATP Binding Cassette, subfamily C (MRP1); *ALOX5:* arachidonate 5-lipoxygenase (5-LO); *EF1α*: eukaryotic translation elongation factor 1 alpha 1; *B2M*: Beta-2-Microglobulin.

Figure Legends

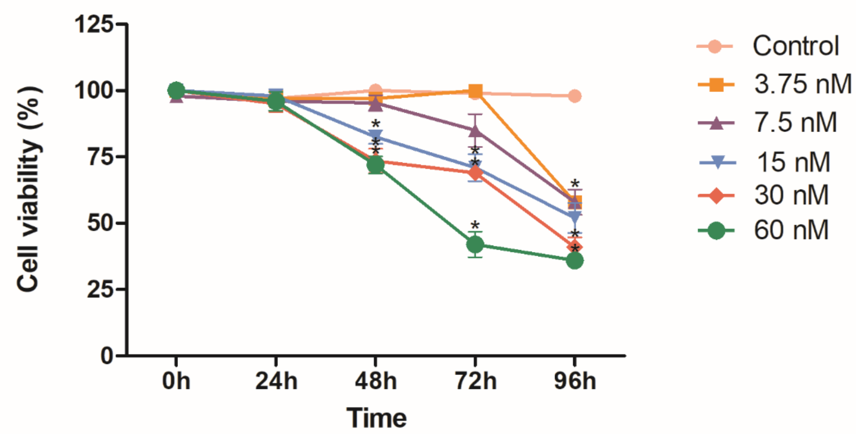

Fig 1: Cell viability after VCR treatments. Analysis immediately (0 h), 24 h, 48 h, 72 h and 96 h after treatment with different concentrations of VCR in K562 cells. Asterisks show significant differences between treatment and control in each time period ($p < 0.05$). Data are expressed as the mean ± standard error. Analyses of variance ANOVA followed by Tukey's *post hoc* test was applied.

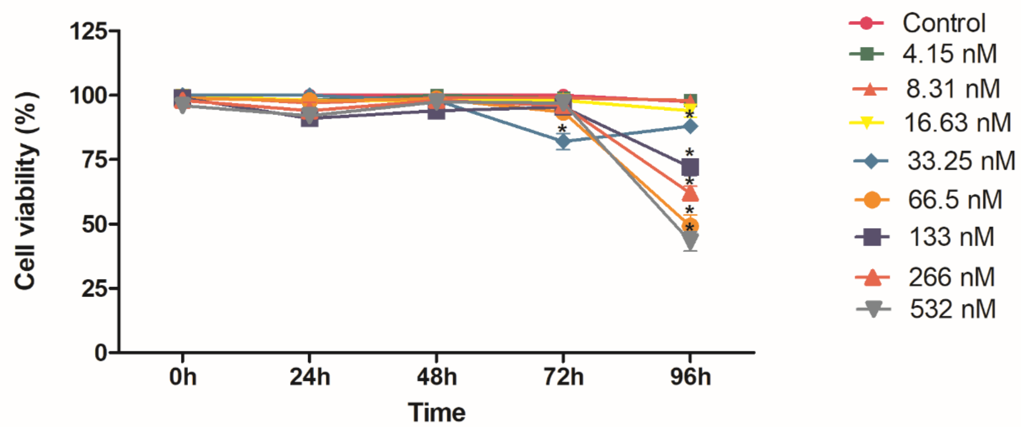

Fig 2: Cell viability after DNR treatments. Analysis immediately (0 h), 24 h, 48 h, 72 h and 96 h after treatment with different concentrations of DNR in K562 cells. Asterisks show significant differences between treatment and control in each time period ($p < 0.05$). Data are expressed as the mean ± standard error. Analyses of variance ANOVA followed by Tukey's *post hoc* test was applied.

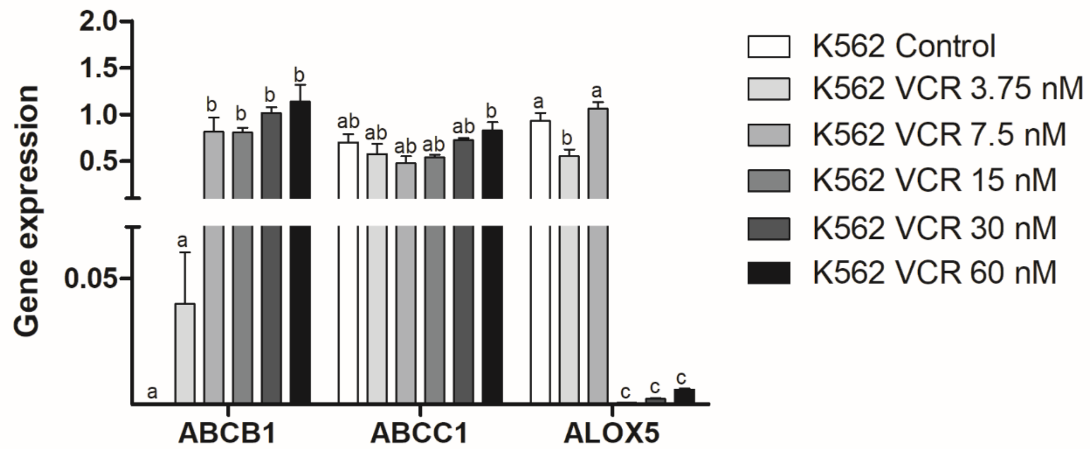

Fig 3: Alteration of gene expression after VCR treatment. Comparison of expression of *ABCB1*, *ABCC1* and *ALOX5* genes between different concentrations of VCR in K562 cells. Different letters indicate significant differences between treatments (p <0.05). Data are expressed as the mean ± standard error. Analyses of variance ANOVA followed by Tukey's *post hoc* test was applied.

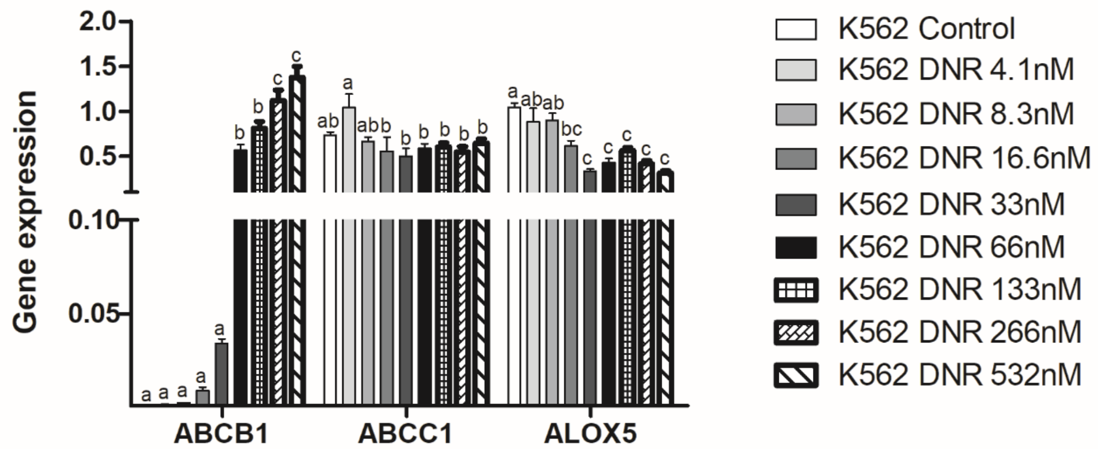

Fig 4: Alteration of gene expression after DNR treatment. Comparison of expression of *ABCB1*, *ABCC1* and *ALOX5* genes between different concentrations of DNR in K562cells. Different letters indicate significant differences between treatments (p <0.05). Data are expressed as the mean ± standard error. Analyses of variance ANOVA followed by Tukey's *post hoc* test was applied.

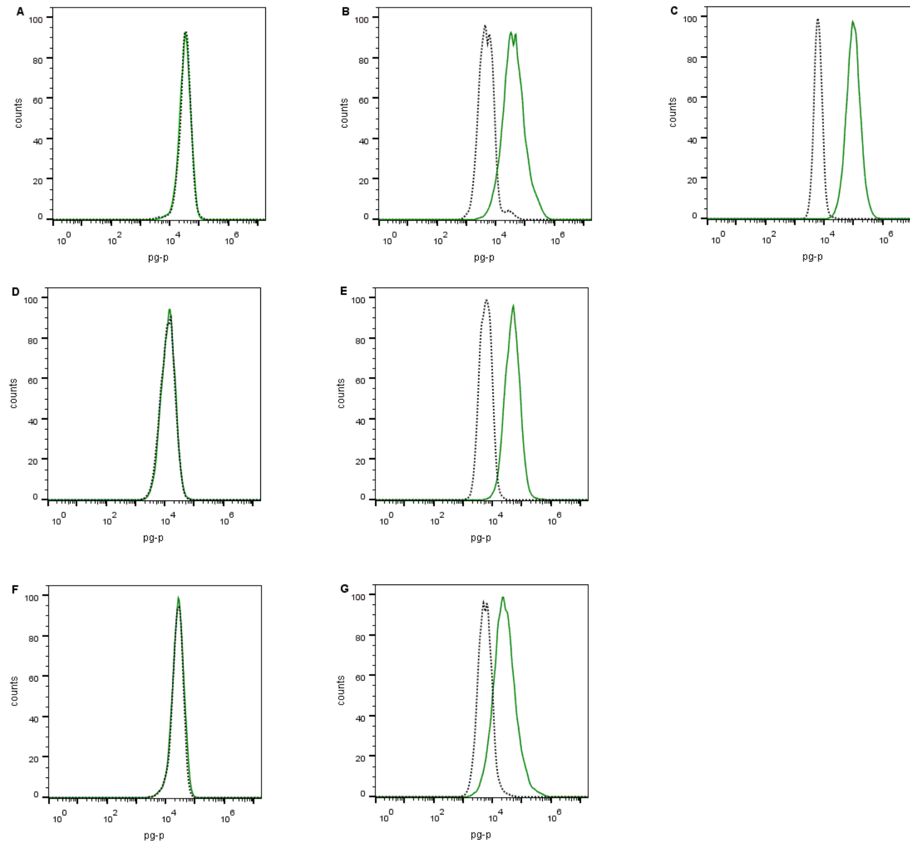

Fig 5: Alteration in ABCB1 protein levels. Representative histogram overlay of cells K562 (A), K562-Lucena (B), FEPS (C), K562 3.75 nM VCR (D), K562 60 nM DNR (E), K562 4.15 nM DNR (F) and K562 532 nM DNR (G), by analyzing ABCB1 protein levels by flow cytometry. Overlapping histograms show CD243 (green line) labeled cells. unmarked cells (dotted line).

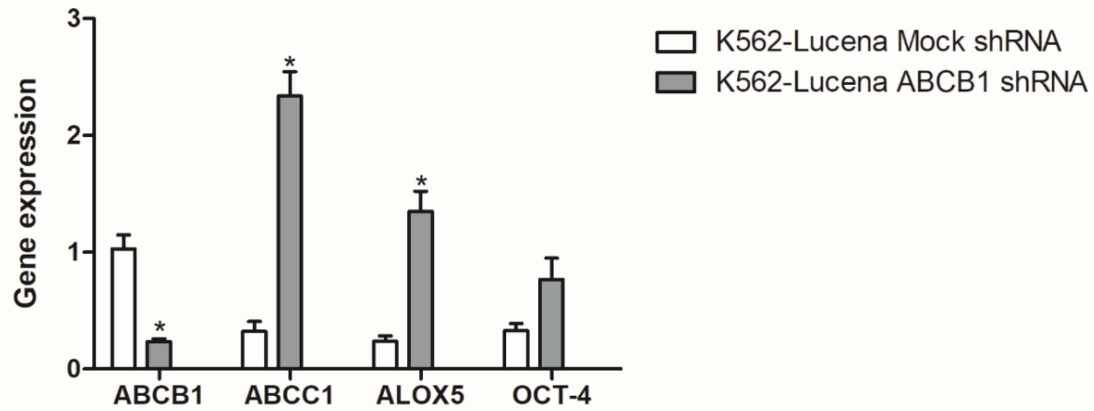

Fig 6: Alteration of gene expression in transfected K562-Lucena cells. Comparison of expression of *ABCB1*, *ABCC1*, *ALOX5* and *OCT-4* genes between K562-Lucena control cells (mock shRNA) and silenced (ABCB1 shRNA). Asterisks show significant differences between the means of each gene (p <0.05). Data are expressed as the mean ± standard error. Student's *t*-test was applied.

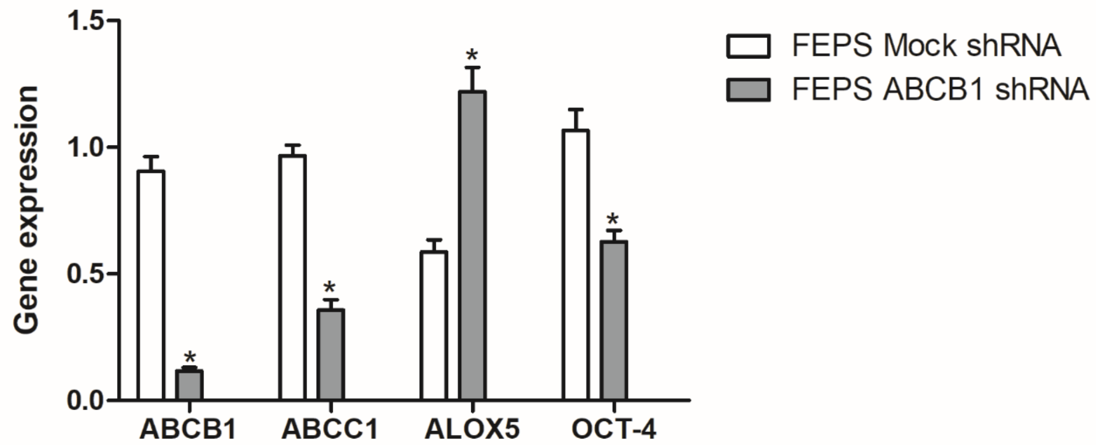

Fig 7: Alteration of gene expression in FEPS transfected cells. Comparison of expression of *ABCB1*, *ABCC1*, *ALOX5* and *OCT-4* genes between FEPS control cells (mock shRNA) and silenced (ABCB1 shRNA). Asterisks show significant differences between the means of each gene (p <0.05). Data are expressed as the mean ± standard error. Student's *t*-test was applied.

DISCUSSÃO GERAL

A resistência a múltiplas drogas (MDR) é uma das principais barreiras para o tratamento do câncer. Nesse cenário, células cancerígenas adquirem o fenótipo MDR caracterizado pela capacidade de superexpressarem a glicoproteína P (P-gp ou ABCB1) na membrana, apresentarem resistência cruzada a certos agentes e diminuirem o acúmulo de drogas no meio intracelular, se tornando insensíveis a drogas (Beck, 1990). Considerando os diferentes mecanismos ativados pelas células cancerígenas em defesa contra os agentes quimioterápicos, novas pesquisas estão sendo realizadas com base na possível relação das células tronco cancerígenas (CTC) na progressão e recidiva do câncer (Nerlov 2009; Chonel & Turhan, 2011; Kreso & Dick, 2014; Cojoc et al., 2015; Villodre et al., 2016).

Neste trabalho, avaliamos pela primeira vez a expressão de *ALOX5* durante a aquisição do fenótipo MDR em linhagem de leucemia mielóide crônica (LMC), bem como a relação do biomarcador de CTC *OCT-4* com a resistência. Para avaliar a influência do pseudogene *OCT4-PG1* na presença do marcador de célula-tronco *OCT-4*, realizamos o silenciamento do pseudogene *OCT4-PG1* na linhagem celular FEPS. Os resultados mostram que as células FEPS silenciadas diminuiram tanto a expressão gênica quanto proteica de OCT-4. As alterações na expressão do gene parental *OCT-4* provavelmente ocorreram a partir de uma regulação pós-transcricional, em que o miRNA (RNA não codificante) do pseudogene pareia com o RNA mensageiros (mRNA) do gene parental regulando sua estabilidade e tradução (Muro et al., 2011). Eventos de regulação do gene parental acontecem nas células porque os pseudogenes sofrem mutações que resultam na síntese de miRNA, os quais regulam outros mRNA específicos estimulando ou inibindo a tradução (Poliseno et al., 2010).

Além de regular características de CT, o fator de transcrição OCT-4 interage com sítios de ligação localizados nas regiões promotoras dos genes *ABCB1*, *ABCC1* e *ABCG2* (Marques et al., 2010). Estes codificam para a síntese de proteínas de membrana com capacidade de extrusão de compostos para o meio extracelular, estando portanto relacionadas ao fenótipo MDR. A alteração dos níveis da proteína OCT-4 no meio intracelular levou a uma diminuição dos níveis de expressão gênica, proteica e atividade de ABCB1. Pesquisas com base clínica mostram que a relação de baixos níveis de expressão de *ABCB1* e altos níveis de *OCT-1* favorecem a um bom prognóstico de pacientes com LMC que se tornaram resistentes ao quimioterápico imatinibe (Vasconcelos et al., 2016). Esta pesquisa reforça a relevância do nosso trabalho em analisar a relação de fatores de transcrição da família OCT com proteínas de extrusão em LMC. No presente trabalho, apesar da diminuição de ABCB1, os níveis de expressão gênica e atividade da proteína ABCC1 aumentaram após o silenciamento do pseudogene *OCT4-PG1*. Este aumento possivelmente aconteceu como um mecanismo compensatório ativado pela linhagem buscando manter o fenótipo MDR.

Com base nesses resultados, tornou-se relevante analisar se a linhagem FEPS, após o silenciamento, se manteria resistente ou não aos quimioterápicos VCR e DNR. As análises com base no número de células viáveis mostraram que as células silenciadas apresentaram sensibilidade a 60 nM de VCR e 532 nM de DNR 72 h após o tratamento, indicando que apesar do aumento na expressão gênica e atividade da proteína ABCC1, as células FEPS silenciadas para o pseudogene *OCT4-PG1* não foram capazes de realizar a extrusão dos quimioterápicos VCR e DNR. É possível que a proteína ABCC1 não tenha sido eficiente na extrusão dos quimioterápicos, pois a linhagem FEPS (não silenciada) apresenta baixa expressão dessa proteína na membrana celular (Daflon-Yunes et al., 2013).

Basicamente, a linhagem FEPS (não silenciada) apresenta uma grande quantidade de proteína ABCB1 ativa na membrana plasmática e resistência aos quimioterápicos VCR e DNR (Daflon-Yunes et al., 2013). Contudo, os dados apresentados neste trabalho mostram que a FEPS silenciada apresentou sensibilidade aos quimioterápicos, possivelmente pela diminuição da expressão e atividade de ABCB1.

As linhagens celulares de LMC estudadas neste trabalho, que apresentam fenótipo MDR, possuem alta expressão dos gene *ABCB1* (K562-Lucena) e alta expressão dos genes *ABCB1* e *ABCC1* (FEPS), quando comparadas a linhagem parental K562 não MDR (Carret-Dias et al., 2016). Outro gene que apresenta expressão diferenciada entre as linhagens é o gene *ALOX5*. Neste caso, existe uma expressão inversa a expressão do gene *ABCB1*, visto que uma superexpressão de *ALOX5* é observada na linhagem K562 (não MDR), uma baixa expressão na linhagem FEPS (MDR) e uma expressão intermediária na linhagem K562-Lucena, quando comparada às linhagens celulares K562 e FEPS. Contudo, uma redução na expressão do gene *ALOX5* foi observada nas linhagens K562 e K562-Lucena após tratamento com fator de diferenciação celular (Carret-Dias et al., 2016).

Além do fator de transcrição OCT-4, também avaliamos o gene *ALOX5* com o propósito de analisar a expressão de genes relacionados a características tronco na linhagem FEPS após o silencimento do pseudogene *OCT4-PG1*. Como esperado, considerando a diminuição na expressão do gene *ABCB1* após silenciamento do pseudogene *OCT4-PG1*, os níveis de expressão do gene *ALOX5* foram aumentados na FEPS silenciada. Resultados semelhantes foram observados quando avaliamos os perfis de expressão gênica em células K562-Lucena e FEPS silenciadas para *ABCB1*. Nestas células, os níveis de expressão gênica de *ABCB1* foram reduzidos, como esperado, enquanto os níveis de expressão do gene *ALOX5* foram superexpressos em ambas

linhagens celulares. Esses dados mostram que as alterações nos perfis de expressão de genes relacionados ao fenótipo MDR podem alterar alguns marcadores relacionados a características de CT.

O gene *ALOX5*, relacionado a capacidade de indiferenciação celular, e sua enzima 5-LO são estudados principalmente nos processos inflamatórios do câncer, porém estudo mostra que a enzima tem a capacidade de induzir a proliferação celular e angiogênese (Park et al., 2012). Umas das vias de sinalização que pode regular esse processo está na capacidade da proteína P53 se conectar a um sítio de ligação do gene *ALOX5* regulando a sua expressão de forma negativa. Essa relação entre *ALOX5* e P53 pode influenciar diretamente na proliferação celular e na apoptose (Gilbert et al., 2015), tornando-se o *ALOX5* um importante alvo para terapia molecular do câncer. Os níveis basais de expressão do gene *p53* são baixos nas linhagens de LMC K562 e K562-Lucena (Carret-Dias et al., 2011). A linhagem celular K562 apresenta uma mutação no gene *p53* (stop codon) o que resulta na ausência de expressão da proteína P53 do tipo selvagem (Cavalcanti Junior et al., 2003) e a expressão de uma proteína P53 mutada Assim, é possível sugerir que a alta expressão de *ALOX5* encontrada na linhagem K562 seja em parte devida à falta de regulação pela proteína P53.

Como forma de analisar a possível relação entre a aquisição do fenótipo MDR e marcadores relacionados a características tronco foi realizada neste trabalho a transformação da linhagem K562 a partir da adição de concentrações graduais dos quimioterápicos VCR ou DNR, objetivando uma superexpressão de *ABCB1* e uma alteração na expressão de *ALOX5* nas células K562. O processo de transformação da linhagem celular K562 exposta a concentrações graduais dos quimioterápicos apresentou, como esperado, um aumento significativo da expressão do gene *ABCB1*. De acordo com os resultados, as células K562 superexpressaram o gene *ABCB1* na concentração de 7,5

nM de VCR, mas o gene *ALOX5* foi suprimido com 15 nM de VCR. Contudo, nenhuma alteração foi observada na expressão do gene *ABCC1.* Estes dados mostram que as células K562 ativaram os mecanismos moleculares para resistência a quimioterapia nas menores concentrações de VCR e, apresentaram alterações relacionadas às características de CT em concentrações intermediárias ao quimioterápico VCR.

Já para exposição a DNR, o gene *ABCB1* foi superexpresso na concentração de 66,5 nM de DNR, enquanto o gene *ALOX5* diminuiu a sua expressão na concentração de 16,6 nM de DNR. Contudo, nenhuma alteração foi observada na expressão do gene *ABCC1.* Estes dados mostram que as células K562 ativam mecanismos moleculares relacionados a características de CT nas menores concentrações de DNR e expressam mecanismos moleculares para resistência a quimioterapia em concentrações intermediárias do quimioterápico DNR. Esses resultados indicam que há uma co-relação na expressão dos genes *ALOX5* e *ABCB1* durante a aquisição do fenótipo MDR.

Na transformação da linhagem K562, não foram considerados o período de tempo longo ou curto da exposição, mas sim a resistência adquirida a cada concentração do quimioterápico adicionada. Similar a este trabalho, as linhagens celulares K562 e KU812 superexpressaram o gene *ABCB1* após exposição aos quimioterápicos Imatinibe e Dasatinibe. No entanto, essas linhagens precisaram de concentrações mais altas para desenvolver a resistência, quando comparado as células K562 expostas ao quimioterápico Doxorrubicina, a qual superexpressou o gene nas concentrações iniciais do quimioterápico (Eadie et al., 2016).

Considerando a superexpressão do gene *ABCB1* nas células K562 após exposição aos quimioterápicos, analisamos a expressão da proteína ABCB1 após exposição às menores e maiores concentrações de VCR e DNR. Como esperado, as células tratadas com as concentrações menores de 3,75 nM de VCR e 4,15 nM de DNR não apresentaram

expressão da proteína. Contudo, após a exposição às maiores concentrações dos quimioterápicos culminando com as concentrações de 60 nM de VCR e 532 nM de DNR, as células apresentaram superexpressão da proteína ABCB1. Esses dados acompanham o observado na superexpressão do gene *ABCB1* nas maiores concentrações. Ademais, as duas maiores concentrações de VCR e DNR determinaram a aquisição do fenótipo MDR nas células K562-Lucena (Rumjanek et al., 1994) e FEPS (Daflon-Yunes et al., 2013).

Como forma de confirmar a co-relação existente entre os genes *ALOX5* e *ABCB1* observada nas células K562 durante a aquisição do fenótipo MDR, analisamos a expressão de genes nas células K562-Lucena e FEPS silenciadas para o gene *ABCB1*. Estas células apresentam redução da expressão do gene *ABCB1* confirmando o silenciamento em ambas as linhagens. As células K562-Lucena e FEPS silenciadas para o gene *ABCB1* apresentaram um aumento significativo na expressão do gene *ALOX5*. Contudo, as células K562-Lucena silenciadas apresentaram um aumento significativo na expressão do gene *ABCC1* enquanto as células FEPS silenciadas apresentaram uma redução na expressão deste gene. Esses dados mostram que, a redução na expressão do gene *ABCB1* que codifica para síntese da proteína ABCB1, altera a expressão do gene *ABCC1* de forma desigual nas células K562-Lucena e FEPS silenciadas. Este perfil de expressão pode ser resultado da ativação de mecanismo compensatório nas células K562-Lucena para manter o fenótipo MDR.

Ao analisar a expressão do gene *OCT-4* em ambas as linhagens silenciadas, não foi observada alteração significativa na expressão deste gene nas células K562-Lucena silenciadas. Já nas células FEPS silenciadas para ABCB1, o gene *OCT-4* apresentou redução na sua expressão. Esta mudança no perfil de expressão gênica pode contribuir para alteração do fenótipo MDR, uma vez que, a proteína OCT-4 se liga a regiões

promotoras dos genes ABCs (Marques et al., 2010), sendo observado uma redução na expressão dos genes *ABCB1* e *ABCC1* nessa linhagem.

Desta forma, é possível sugerir que durante a indução do fenótipo MDR nas células K562 tratadas com os quimioterápicos VCR e DNR, a expressão de *ABCB1* e *ALOX5* é inversamente proporcional, sendo a co-relação entre estes dois genes confirmada. Além disso, , é possível especular que o tratamento quimioterápico não induz a transição de células somáticas tumorais para CTCs, mas que provavelmente as CTCs sejam as responsáveis pelo desenvolvimento do fenótipo MDR no câncer. Essa condição é viável uma vez que estas células em sua divisão celular geram células filhas com o fenótipo MDR e ainda, podem transferir para células vizinhas vesículas exossomais com miRNAs e proteínas induzindo a resistência (Azmi et al., 2013).

CONCLUSÕES

- ✓ O silenciamento do pseudogene *OCT4-PG1* alterou a expressão do gene e da proteína OCT-4. Como consequência, alterou as proteínas relacionadas ao fenótipo MDR.

- ✓ As células FEPS silenciadas para o pseudogene *OCT4-PG1* apresentaram sensibilidade aos quimioterápicos VCR e DNR.

- ✓ Ao adquirir resistência aos quimioterápicos, as células cancerígenas podem ativar mecanismos moleculares como a via do gene *ALOX5*.

- ✓ O gene *ALOX5* apresentou maior expressão quando a expressão do gene *ABCB1* , relacionado ao fenótipo MDR, estava diminuída.

- ✓ A transformação da linhagem celular K562 a partir dos quimioterápicos VCR e DNR estimulou o aumento na expressão do gene *ABCB1* e diminuição da expressão do gene *ALOX5* em concentrações diferentes,demosntrando a existência de uma co-relação entre estes genes.

- ✓ O silenciamento do pseudogene OCT4-PG1 e a transformação da linhagem celular K562 comprovaram a relação inversa na expressão dos genes *ABCB1* e *ALOX5* nas três linhagens celulares estudadas.

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