UNIVERSIDADE DE SÃO PAULO

FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

PEDRO LUIZ PORFIRIO XAVIER

Exploring Epigenetic Mechanisms to Target Mammary Cancer Cells: A Comparative Approach

Pirassununga

2020

PEDRO LUIZ PORFIRIO XAVIER

Exploring Epigenetic Mechanisms to Target Mammary Cancer Cells: A Comparative Approach

Versão Corrigida

Tese apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências do programa de Pós-graduação em Biociência Animal.

Área de Concentração: Biologia celular e molecular

Orientador: Prof. Dr. Heidge Fukumasu

Pirassununga

2020

Ficha Catalográfica

Ficha catalográfica elaborada pelo
Serviço de Biblioteca e Informação, FZEA/USP,
com os dados fornecidos pelo(a) autor(a)

X3e

Xavier, Pedro Luiz Porfirio
Exploring Epigenetic Mechanisms to Target Mammary Cancer Cells: A Comparative Approach / Pedro Luiz Porfirio Xavier ; orientador Heidge Fukumasu. -- Pirassununga, 2020.
185 f.

Tese (Doutorado - Programa de Pós-Graduação em Biociência Animal) -- Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo.

1. auto-renovação. 2. câncer de mama. 3. deacetilases de histonas. 4. epigenética. 5. oncologia comparada. I. Fukumasu, Heidge , orient. II. Título.

Certificado CEUA

Viemos através deste, certificar que as linhagens oriundas de carcinomas mamários caninos, utilizadas nos experimentos dessa tese, estão protocoladas sob o CEUA 3094061014, vigente desde 06/10/2014.

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

São Paulo, 08 de julho de 2020
CEUA N 3094061014

Ilmo(a). Sr(a).
Responsável: Heidge Fukumasu
Área: Patologia

Título da proposta: "EXPRESSÃO GÊNICA GLOBAL ESPECÍFICA DE CÉLULAS INICIADORAS DE TUMOR EM NEOPLASIAS MAMÁRIAS DE CADELAS".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 006558)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 17/junho/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Prezados bom dia, O projeto foi sendo prorrogado pois um segundo experimento de doutorado tem sido realizado dentro da mesma temática e com as mesmas amostras de linhagens de células isoladas inicialmente neste mesmo projeto. Ressalto que apenas células já isoladas tem sido utilizadas. Sem o uso de novos animais. Desde já muito obrigado e até logo, ".

Comentário da CEUA: "Solicitado uma extensão do prazo, pois um novo projeto de doutorado está sendo conduzido, o pesquisador informa que não foram utilizados novos animais, apenas células já coletadas previamente".

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenador
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

FOLHA DE APROVAÇÃO

Nome: Pedro Luiz Porfirio Xavier

Título: Exploring Epigenetic Mechanisms to Target Mammary Cancer Cells: A Comparative Approach

Tese apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências do programa de Pós-graduação em Biociência Animal.

Área de Concentração: Biologia celular e molecular

Data:_____/_____/_____

BANCA EXAMINADORA

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

DEDICATÓRIA

No ontem, no hoje e no sempre, todas as minhas conquistas serão dedicadas a aqueles que me permitiram conquistá-las. Meus pais, João Batista Xavier e Cleuza Porfirio da Silva Xavier.

AGRADECIMENTOS

A minha imensa família. Vocês são minha base de amor, carinho e felicidade. Obrigado por todos os domingos juntos, almoçando, bebendo cerveja e contando histórias. Sem vocês, nenhuma destas linhas fariam sentido.

Ao meu orientador e amigo, Professor Heidge Fukumasu. Serei eternamente grato por todos os ensinamentos biológicos, conselhos e amizade. Por nunca medir esforços para tornar nosso grupo sempre e sempre melhor. Um exemplo de liderança e profissionalismo. Por me oferecer liberdade de pensamento. Obrigado por sempre dizer a frase "Eu não sei. Vai estudar, depois a gente conversa." Na maioria das vezes é lógico que ele sabia, mas se ele me contasse, qual seria a graça? Obrigado por tudo.

Ao doutor George Shigueki Yasui, que sempre estará em todos os meus agradecimentos científicos. Por ter me feito gostar de ciência de verdade e por nossas colaborações que perduram até hoje. São frases e detalhes suscintos que fazem diferença na vida de uma pessoa. A frase desse cara pra mim, em 2013, no final da minha graduação, foi a seguinte: "Pedro, eu acho que você tem um perfil para a pesquisa e um grande potencial. Continue assim." Isso fez toda a diferença.

A Dra. Susanne Müller, também um exemplo de pesquisadora e profissional, que me abriu as portas para a maior oportunidade da minha vida durante minha BEPE. Serei eternamente grato pelos ensinamentos e ajuda. E a todo o grupo do Instituto de Farmacêutica Química e do BMLS da Universidade de Goethe, especialmente ao Dr. Stefan Knapp que abriu a porta de seu laboratório para os meus experimentos. E a todo o imenso grupo, por me acolherem tão bem e me ajudarem muito durante os 8 meses que trabalhei por lá.

A FZEA, ao programa de Biociência Animal e a todos os professores, pesquisadores e funcionários que dele fazem parte. Obrigado pela oportunidade!

Aos professores Ricardo F. Strefezzi, Juliano C. Silveira, Debora A.P.C Zuccari, Fabiana F. Bressan, Flávio Meirelles, Edson R. da Silva, Bruno H. Saranholi, José B. S. Ferraz e Pedro M. Galetti Jr. pela ajuda com os experimentos e disponibilização de seus equipamentos e laboratórios.

Aos Lobos, Bruno, Felipe, Jefferson e Raphael. Os maiores amigos que a vida poderia me conceder. Mais de 20 anos de uma amizade extremamente valiosa em minha vida.

Ao V.N.A, meus irmãos! Ary Neto, Birna, Chupeta, Gui, JP, Digo, Fabinho, Narêz, Banana, Lupercim, Mathias, Mirtão, Pepito, Marcão, Pg, Sarapa, Tibúrcio, Urso.

Aos amigos que fiz no LOCT nesses 7 anos. Especialmente a Yô, Nina, Porco, Gabi Ribeiro, Chico, Pedrinho, Pâmelas, Tais, Jéssika e Lídia. Quantas coisas já passamos juntos. Desde a ida ao bandeco, os churrascos e comemorações... Como foi bom poder partilhar esse tempo com vocês! E ao Professor Ricardo Strefezzi por toda a amizade, um exemplo de Professor, pesquisador, didática e profissionalismo, nos ajudando sempre para o avanço de nossos projetos. Um orgulho imenso de vocês!

A todos os amigos que a FZEA e Pirassununga me deram. São tantos que fica complicado citar todos os nomes aqui. Mas em especial aos meus amigos de rep Flufly, Mifode,

Birruga, Porco, Tio Chico, Alois, Stroguinho, Miagui e Gui. Também aos amigos do LMMD: Rafão, Ju, Ramon Jovem, Strepa, Léo, Deba, Gabriel, Matata, Láis e Aninha. E aos de banda Dan, Gus, Poletera e Endrigueta. Passamos por vários churrascos e festas juntos e tornaram essa cidade extremamente especial para mim.

Aos membros da banca examinadora pelo tempo precioso no julgamento desse trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

A Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) por todo o suporte financeiro para a execução desses trabalhos e pelas bolsas (processos 2017/11966-4 e 2019/05778-6.

"Jack, nós temos que ir e nunca parar de

seguir enquanto a gente não chegar lá."

"Para onde estamos indo, cara?"

"Eu não sei, mas temos que ir."

"Não tínhamos que falar mais nada. O único a fazer era ir."

"A estrada deve guiar-nos por todo o mundo."

Jack Kerouac – On the Road

RESUMO

XAVIER, P.L.P. **Explorando Mecanismos Epigenéticos Contra Células de Câncer de Mama: Uma Abordagem Comparada.** 185F. Tese (Doutorado). Faculdade de Zootecnia e Engenharia de Alimentos. Universidade de São Paulo.

O câncer é uma das principais causas de morte em cães no mundo e as neoplasias mamárias representam mais de 50% dos tumores diagnosticados em fêmeas. Além disso, as neoplasias mamárias apresentam uma série de similaridades clínicas e moleculares com o câncer de mama em mulheres, destacando-as como excelentes modelos para o estudo da oncologia comparada. Os mecanismos epigenéticos são responsáveis por regular a expressão gênica nas células e desregulações nesses mecanismos estão associadas ao desenvolvimento de diversos tipos de câncer tanto em humanos como em cães. Assim, a utilização do modelo canino pode ser importante para o desenvolvimento de potenciais terapias epigenéticas para o câncer, em específico para o câncer de mama. Assim, essa tese teve como principais objetivos: 1) revisar os mecanismos epigenéticos que estão por trás do desenvolvimento de diferentes tipos de câncer em cães, comparando-os com as anormalidades já bem descritas no câncer humano; 2) determinar possíveis alvos moleculares associados aos processos de tumorigenicidade e invasão de células de câncer de mama canino (Células CMC); 3) determinar potenciais alvos epigenéticos associados ao potencial de tumorigenicidade e auto-renovação de células de câncer de mama canino; 4) observar os efeitos de um novo inibidor duplo de proteínas epigenéticas BET e deacetilases de histonas (HDACs) em células de câncer de mama humano utilizando modelos *in vitro* 2D e 3D. No primeiro capítulo, reunimos uma série de informações demonstrando que algumas modificações epigenéticas são similares no câncer em humanos e caninos, sugerindo uma potencial abordagem utilizando o modelo canino para determinar novos mecanismos epigenéticos reguladores do câncer e para o desenvolvimento de novas terapias. No capítulo 2, utilizando modelos *in vitro*, demonstramos que os fatores de transcrição associados ao processo de transição epitélio-mesenquimal (EMT), ZEB1 e ZEB2, estão associados ao potencial de tumorigenicidade e invasão de células CMC e são mais expressos em células CMC com fenótipo mesenquimal do que células CMC com fenótipo epitelial. Além disso, observamos que vias moleculares associadas aos processos de EMT, invasão e tumorigenicidade, como organização e degradação de matriz extracelular, adesão focal, sinalização de TGF-β/BMP, PI3K-AKT e WNT são enriquecidas em genes superexpressos nas células CMC com fenótipo mesenquimal. Além disso, esse trabalho foi importante para a determinação das linhagens com maior potencial tumorigênico no nosso banco de células para a utilização no capítulo 3. Já no capítulo 3, nós demonstramos que a inibição de uma família de proteínas "readers" epigenéticas, conhecidas como proteínas BET, pelo inibidor (+)-JQ1, é uma estratégia promissora para inibir os fenótipos de tumorigenicidade e auto-renovação de células CMC, em modelos *in vitro*. A inibição das proteínas BET resultou em diminuição da formação de tumoresferas em placas de baixa aderência e de colônias no ensaio de ágar-mole, dois modelos *in vitro* utilizados para se estudar auto-renovação e tumorigenicidade. Além disso, inibição de proteínas BET resultou em diminuição da expressão de importantes genes associados a vias moleculares de auto-renovação como WNT, NOTCH, Hedgehog e PI3K/AKT/mTOR, e diminuiu a expressão de ZEB2, o qual demonstramos ser importante para o fenótipo de tumorigenicidade em células CMC no capítulo 2. Finalmente, no capítulo 4 determinamos que o inibidor duplo de proteínas BET/HDACs, TW09, reduziu a viabilidade celular e aumentou a morte celular em células de câncer de mama humano em comparação com as inibições individuais de proteínas BET e HDACs. Além disso, TW09 diminuiu a formação de tumoresferas primárias e

secundárias demonstrando efeito sobre os potenciais de tumorigenicidade e auto-renovação das células de câncer de mama. Sendo assim, TW09 demonstrou bons efeitos nessas células. Ainda pretendemos aprofundar ainda mais os estudos dos efeitos da inibição dupla de proteínas BET/HDACs por TW09 no câncer de mama, em células humanas e caninas, podendo confirmar essa estratégia como uma potencial terapia para o câncer de mama em ambas espécies. Por fim, os resultados demonstrados ao longo dos capítulos dessa tese colaboram para a confirmação e descoberta de mecanismos importantes para o desenvolvimento do câncer de mama em cães e seus fenótipos, além de ressaltarem o modelo canino como um bom modelo para o estudo da oncologia comparada e busca de novos alvos e terapias contra o câncer, já que vários dos mecanismos e efeitos observados nesse modelo, corroboram com aqueles vistos em células de câncer humano.

Palavras-Chave: auto-renovação, câncer de mama, deacetilases de histonas, epigenética, oncologia comparada, proteínas BET, tumorigenicidade

ABSTRACT

XAVIER, P.L.P. **Exploring Epigenetic Mechanisms to Target Mammary Cancer Cells: A Comparative Approach.** 185p. PhD Thesis. Faculty of Animal Science and Food Engineering. University of São Paulo. 2020.

Cancer is one of the main causes of deaths in dogs worldwide, and mammary cancer in female dogs corresponding to more than 50% of all types of tumors. In addition, mammary tumors in dogs share many clinical and molecular similarities in comparison to breast cancer in women. Therefore, dogs are great models for comparative studies in oncology. Epigenetic mechanisms are key regulators of gene expression and dysregulation of these mechanisms are associated to the development of several types of cancer both in humans and dogs. Thus, canine model may be important for the development of innovative and potential therapies for cancer, especially for breast cancer. Thus, the main purposes of this thesis were: 1) to review the epigenetic mechanisms behind the development of different types of cancer in dogs, in comparison with abnormalities well described in human cancer; 2) to determine possible molecular targets associated with tumorigenicity and invasiveness in canine mammary cancer cells (CMC cells); 3) to determine potential epigenetic targets associated with self-renewal and tumorigenicity in CMC cells; 4) to observe the effects of a new dual BET/HDAC inhibitor in human breast cancer cells using 2D and 3D *in vitro* models. In the first chapter, we gathered findings demonstrating that some epigenetic abnormalities are similar in both human and canine cancer, suggesting a potential approach to use the canine model to determine new epigenetic mechanisms regulating cancer and to develop new therapies. In chapter 2, using *in vitro* models, we demonstrate that epithelial-mesenchymal (EMT)-associated transcription factors, ZEB1 and ZEB2, are associated with tumorigenicity and invasiveness of CMC cells, exhibiting higher expression in mesenchymal-like cells in comparison with epithelial-like cells. In addition, we observed that molecular pathways associated with EMT, invasiveness, and tumorigenicity, such as ECM organization and degradation, focal adhesion, TGF-β / BMP signaling, PI3K-AKT signaling, and WNT signaling are enriched to upregulated genes overexpressed in mesenchymal-like cells. Furthermore, this work was important to determine cell lines presenting higher tumorigenic potential in our cell bank. In chapter 3, we demonstrated that inhibition of a family of epigenetic reader proteins, known as BET proteins, by (+)-JQ1, is a promising strategy to suppress *in vitro* tumorigenicity and self-renewal of CMC cells. BET inhibition resulted in a decrease of tumorspheres formation in low-adherence plates and colony formation in soft-agar assay, classical models used to assess *in vitro* self-renewal and tumorigenicity. Furthermore, inhibition of BET proteins decreased expression of important genes associated with self-renewal pathways including WNT, NOTCH, Hedgehog and PI3K/AKT/mTOR. Besides that, BET inhibition decreased *ZEB2* expression, a transcription factor important for the maintenance of self-renewal in CMC cells. Finally, in chapter 4, we observed that the dual BET/HDAC inhibitor, TW09, reduced cell viability and increased cell death in human breast cancer cells in comparison with individual inhibition of BET and HDAC proteins by (+)-JQ1 and CI994, respectively. In addition, TW09 reduced number of primary and secondary BC tumorspheres, suggesting effects regarding tumorigenicity and self-renewal potential of breast cancer cells. Thus, TW09 demonstrated good effects in these cells. We still intend to deeply study the effects of dual BET/HDAC inhibition by TW09 on breast cancer both

in human and canine cells in order to confirm this strategy as a potential and innovative therapy for mammary cancer in both species. Finally, the results demonstrated in this thesis collaborate to the validation and discovery of important mechanisms behind mammary cancer and its phenotypes in dogs, besides highlight the canine model as a great model to perform comparative studies in oncology and search for new targets and therapies for cancer, since several of the mechanisms and effects observed in this model, corroborate those observed in human cancer cells.

Keywords: BET proteins, comparative oncology, epigenetics, histone deacetylases, mammary cancer, self-renewal, tumorigenicity

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS AND ACRONYMS

5-azaC – 5-azacytidine

5-azadC - 5-aza-2′-deoxycytidine

AML – Acute Myeloid Leukemia

ANOVA – Analysis of Variance

AO/EB assay – Acridine Orange/Ethidium Bromide assay

ATAC-Seq – Assay for Transposase Accessible Chromatin Sequencing

BB-CLA – BB-CL-Amidine

BET – Bromodomain and Extraterminal

BSA – Bovine Serum Albumin

CAMs – Cell Adhesion Molecules

CCOGC – Canine and Comparative Oncology and Genomics Consortium

cDNA – Complementary DNA

CFS barrier – Cerebrospinal barrier

CGIs – CpG islands

CMCs – Canine Mammary Cancer

CMML - Chronic Myelomonocytic Leukemia

COBRA – Combined Bisulfite restriction analysis

COI – Cytochrome Oxidase I

CPM – Counts Per Million

CSCs – Cancer Stem Cells

CT – Cycle Threshold

DAPI - 4',6-Diamidino-2-Phenylindole

DE – Differential Expression

DMSO - Dimethyl sulfoxide

DNMTs – DNA Methyltransferases

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EMT – Epithelial-Mesenchymal Transition

ENCODE – Encyclopedia of DNA Elements

EP cells – Epithelial-like cells

ERα – Estrogen Receptor α

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FDR – False Discovery Rate

FGF – Fibroblast Growth Factor

GTEx – Genotype-Tissue Expression

HATs – Histone Acetyltransferases

HDACi – Histone Deacetylase Inhibitors

HDACs – Histone Deacetylases

HKDMs – Histone Demethylases

HKMTs – Histone Lisine Methyltransferases

HMC – Human Mammary Cancer

HRP antibody – Horseradish Peroxidase antibody

HR-positive tumors – Hormone Receptor-positive tumors

iBETs – BET inhibitors

IC_{50} - Half Maximal Inhibitory Concentration

IGF – Insulin-like Growth Factor

IgG – Immunoglobulin G

IMPC – Invasive Micropapillary Carcinoma

KEGG – Kyoto Encyclopedia of Genes and Genomes

lncRNAs – Long Non-Coding RNAs

MDS – Myelodysplastic Syndrome

ME cells – Mesenchymal-like cells

miRNAs – MicroRNAs

MSP – Methylation Specific PCR

MTT - (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NHL – Non-Hodgkin Lymphoma

NIH – National Institute of Health

OS – Osteosarcoma

PADs – Protein-Arginine Deiminases

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

PHD finger – Plant Homeodomain finger

PI – Propidium Iodide

PMS - N-methyl dibenzopyrazine methyl sulfate

PR – Progesterone Receptor

PRMTs – Protein Arginine Methyltransferases

PS – Phosphatidylserine

RIN – RNA Integrity Number

RIPA buffer – Radioimmunoprecipitation Assay buffer

RISC – RNA-Induced Silencing Complex

RNA-Seq – RNA Sequencing

RT – Room Temperature

SAHA – Suberoylanilide Hidroxamic Acid

SDS-PAGE - Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SGC – Structural Genomic Consortium

TBST - Tris-Buffered Saline Tween

TCGA – The Cancer Genome Atlas

TFs – Transcription Factors

TNBC – Tryple Negative Breast Cancer

TSA – Trichostatin A

UTR – Untranslated Region

VPA – Valproic Acid

XTT - (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide)

SUMMARY

1. Introduction

Dogs are the human's best friend and are genuinely becoming part of the human family. Based on this sentence, more efforts to develop the field of veterinary care arise. Consequently, life expectancy of these animals increases and age-related diseases, including cancer, are usually diagnosed, becoming the main cause of death worldwide in dogs. Mammary cancer corresponds to half of diagnosed tumors in female dogs and share a series of clinical and molecular similarities with breast cancer in women, such as hormonal dependence, age of onset, and molecular alterations. Thus, dogs are a great valuable model for comparative oncology, enhancing the search for diagnostic and prognostic markers and innovative strategies and targets to therapy. Our group has been working with naturally occurring canine cancer such as mammary cancer and lymphoma and our main goals are to determine and characterize new therapeutic targets studying intra- and inter-heterogeneity of complex tumors; new tools to improve the diagnosis, prognosis and prediction of treatment response as the use of liquid biopsies; and new therapies as neoantigen vaccines, oncolytic viruses, and epigenetic modulators.

Epigenetic mechanisms regulate gene expression and are essential for normal development and homeostasis. Thus, modifications in the epigenetic landscape dysregulate gene expression and are a hallmark of cancer. However, differentially of genetic alterations, epigenetic modifications can be reversible opening the possibility to treat cancer targeting defective epigenetic proteins. In dogs, epigenetic modifications are also driving the development of different types of cancer and many of these abnormalities are similar in both human and canine species as we highlight in the first chapter of this thesis. In addition, advancement of knowledge and technologies of genomic and epigenetic tools support a potential approach using the canine model to determine new epigenetic mechanisms regulating cancer, diagnostic/prognostic markers and targets for the development of new anticancer drugs.

In this Thesis, we elaborate four articles. The first one describes the epigenetic mechanisms in canine cancer, comparing the abnormalities found in their respective types of human cancer. On the second article, we observed genes and signaling pathways that possibly regulate tumorigenicity and invasiveness in canine mammary cancer cells and, consequently, are potential therapeutic targets to the disease. Next, on the third article we performed a screening of an epigenetic inhibitor library using 3D *in vitro* models to

determine a family of epigenetic proteins (BET proteins), belonging to the class of epigenetic readers, as potential targets to suppress self-renewal and tumorigenicity in canine mammary cancer cells. Finally, in the last article, we presented the first experiments of a new dual BET/HDAC inhibitor (TW09) test in human breast cancer cells and its effects. We still intend to perform substantial assays to conclude and determined TW09 as a potential and innovative strategy to treat mammary cancer in both human and canine species, improving the acknowledgement regarding epigenetic therapies in the field of comparative and translational oncology.

2. Chapter 1: Epigenetic Mechanisms in Canine Cancer

Pedro L. Porfirio Xavier [1], Susanne Müller [2], Heidge Fukumasu [1]

[1] Laboratory of Comparative and Translational Oncology (LOCT), Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, Brazil

[2] Structural Genomics Consortium and Institute of Pharmaceutical Chemistry, Buchmann Institute for Molecular Life Sciences, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

Published in Frontiers in Oncology, doi: 10.3389/fonc.2020.591843, Oct 2020

Abstract

A plethora of data has highlighted the role of epigenetics in the development of cancer. Initiation and progression of different cancer types are associated with a variety of changes of epigenetic mechanisms, including aberrant DNA methylation, histone modifications, and miRNA expression. At the same time, advances in the available epigenetic tools allow to investigate and reverse these epigenetic changes and form the basis for the development of anticancer drugs in human oncology. Although human and canine cancer shares several common features, only recently, studies emerged investigating the epigenetic landscape in canine cancer and applying epigenetic modulators to canine cancer. This review focuses on the existing studies involving epigenetic changes in different types of canine cancer and the use of small-molecule inhibitors in canine cancer cells.

Keywords: canine cancer, comparative oncology, DNA methylation, epigenetics, histone modifications, non-coding RNAs

2.1 Introduction

The epigenome consists of a set of complex, dynamic, and reversible information comprising chemical modifications of the DNA and histone proteins, which are directly associated with the regulation of gene expression within the genome. These modifications, described as “Epigenetic Marks”, are heritable and occur without changes in the DNA sequence, playing a key role in biological processes such as embryonic development, differentiation, gene imprinting and silencing of the X chromosome. Furthermore, epigenetic modifications can affect DNA accessibility having a major

influence on DNA-based processes including transcription, DNA repair, and replication (BAYLIN; JONES, 2011).

The epigenetic process is driven by a machinery of proteins, responsible for adding, removing or recognizing modifications of the DNA and histones. Epigenetic 'writers' are responsible for adding epigenetic marks and include DNA and histone methyltransferases as well as histone acetyltransferases (HATs); the 'erasers' such as histone deacetylases (HDACs) and histone demethylases (HKDMs) remove the corresponding epigenetic marks. Finally, a class of proteins exists, which recognizes and 'interprets' epigenetic modifications, referred to as 'readers', a large class of proteins with reader domains for residues such as acetyllysine residues, including bromodomains, and methyllysine residues, such as Chromodomains, Tudor Domains, PhD domains and others (BISWAS; RAO, 2018) (**Figure 1**). All these proteins play important functions in regulating gene expression, acting directly on DNA accessibility or indirectly recruiting non-coding RNAs and chromatin remodelers (DAWSON, 2017). Therefore, abnormal expression or mutations in these chromatin regulators can alter the pattern of gene expression and, consequently, lead to the induction and maintenance of diverse types of diseases, including cancer. Besides, these epigenetic alterations represent disease biomarkers with diagnosis and/or prognosis potential (DAWSON; KOUZARIDES, 2012). Several of these epigenetic protein classes have been shown to contain druggable targets opening up possibilities to treat epigenetic-associated diseases (HUSTON et al., 2015; SCHEER et al., 2019; WU et al., 2019).

The dog is probably the best model for human disease and has several advantages in comparison to other animal models, such as natural development of several different tumors similar to humans; generally shares the same environment and exposure to the same carcinogens as humans thereby influencing the epigenetic make-up (KHANNA et al., 2006). However, in comparison to human cancer, the number of studies investigating the epigenetic landscape in canine cancer is still restricted and the potential of epigenetic drugs in the treatment of canine cancer remains widely unexplored. In this review, we highlight epigenetic studies of canine cancer and provide information about how alterations of epigenetic regulators can influence diverse types of canine cancer. We also highlight potential drugs aimed at targeting these epigenetic regulators.

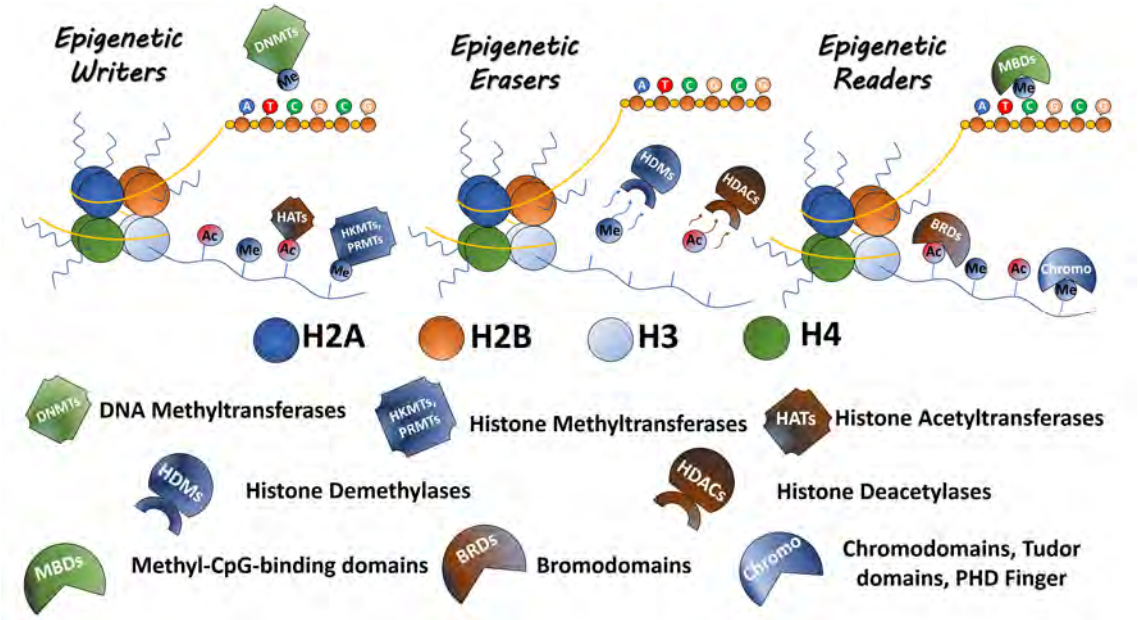


Figure 1. Epigenetic modulators. The epigenetic proteins are classified as Readers, Writers and Erasers. Writers including DNMTs, HKMTs, PRMTs, and HATs are responsible to mark residues in DNA or histone tails. Erasers, including HKDMs and HDACs, remove epigenetic marks. Readers such as proteins containing bromodomains, chromodomains, Tudor domains and PHD fingers recognize and bind to the epigenetic marks.

2.2 An overview of canine cancer

Over the past years, the animal-owner relationship has been changing and pets have genuinely become part of the human family. Therefore, advances in veterinary care have emerged, and, just like in humans, an increased life expectancy of dogs is observed (COZZI et al., 2017). Consequently, age-related diseases, mainly cancer, are becoming the main causes of deaths in dogs worldwide (EGENVALL et al., 2005; FIGHERA et al., 2008; ADAMS et al., 2010; FLEMING; CREEVY; PROMISLOW, 2011; GRÜNTZIG et al., 2015; INOUE et al., 2015). The epidemiologic studies of canine cancer are largely retrospective and usually present varying results depending on the region where they were performed. The incidence of cancer in dogs is 99.3 per 100,000 in male dogs and 272.1 in female dogs according to a study performed in Genoa, Italy (MERLO et al., 2008). A large set of studies has shown that most common types of canine tumors are located in the skin followed by mammary tumors. Some studies have registered skin tumors frequencies as high as 40% or 50% (BRØNDEN et al., 2010; GARCÍA et al., 2019). However, depending on the proportion between females and males, mammary tumors have been observed around in about 36% of total cases (CHOI; YOON; JEONG, 2016),

since mammary tumors account for more than 50% of the diagnosed tumors in females (SORENMO, 2003; GUPTA, 2012; BAIONI et al., 2017; PASTOR et al., 2018). Other common types of cancer in dogs are located in the soft tissues (GRÜNTZIG et al., 2015), hematopoietic, and lymphoid tissues (BRØNDEN et al., 2010), digestive organs (CHOI; YOON; JEONG, 2016), and bones (FENGER; LONDON; KISSEBERTH, 2014).

Most canine cancer share a common pattern with the corresponding human disease including incidence, spontaneous development, associated risk factors, response to treatment, and expression of molecular targets. Non-Hodgkin Lymphoma (NHL), for example, presents an incidence between 15.5 and 29.9 per 100,000 in humans and 15-30 per 100,000 in dogs (GARDNER; FENGER; LONDON, 2016), while mammary tumors incidence in female dogs is around 25% compared to 12% in women. Interestingly, some studies even showed that the average age at onset of mammary tumors is approximately the same for women and female dogs and the peak incidence to mammary cancer is comparable if their age is calculated proportionally (STRANDBERG; GOODMAN, 1974; QUEIROGA et al., 2011). Some strategies for cancer treatment can be applied to humans and dogs. CHOP therapy (vincristine, cyclophosphamide, prednisone, and doxorubicin), for instance, exhibits favorable outcomes in patients with lymphoma in both species, with median survival times of 8-13 months for dogs (CHUN, 2009; REBHUN et al., 2011).

Regarding genetic and signaling-pathway alterations, many types of canine cancer show similarities with their respective types of cancer in humans. Both human and canine osteosarcoma tumors carry mutations in tumor suppressor genes such as *p53* (VAN LEEUWEN et al., 1997; KIRPENSTEIJN et al., 2008; FENGER; LONDON; KISSEBERTH, 2014) and *RB1* (THOMAS et al., 2009), besides alterations in oncogenes expression including *MYC* and *MET* and constitutive expression of *STAT3* (FOSSEY et al., 2009; FENGER; LONDON; KISSEBERTH, 2014). Overexpression of *MYC*, a consequence of copy number aberrations, can also be observed in both human and canine lymphomas (THOMAS et al., 2011; POPHALI et al., 2020). Diffuse large B-Cell Lymphoma in both species exhibit alterations in NF-κB and B-cell receptors pathway signaling (MUDALIAR et al., 2013). In leukemia, a classical chromosomal rearrangement on the Philadelphia Chromosome is present in 95% of cases, producing a constitutively active cytoplasmic tyrosine kinase fusion protein, BCR-ABL (QUINTÁS-

CARDAMA; CORTES, 2009). Although less constant, the same translocation was observed in dogs with leukemia (CRUZ CARDONA et al., 2011; FIGUEIREDO et al., 2012). Canine mammary tumors and breast cancer in women share many clinical and molecular similarities, such as hormonal dependence, age of onset, and identical course of the disease (GUPTA; SOOD; UPPAL, 2012). At the molecular level, the disease in both species also exhibits equivalent features. Despite not having a consistent molecular classification based on specific molecular markers (estrogen receptor, progesterone receptor, and *HER2*) like in breast cancer, canine mammary tumors can present germline mutations in *BRCA1* and *BRCA2* (RIVERA et al., 2009), important tumor suppressor genes inherited mutated in women breast tumors (FORD et al., 1998). Likewise, overexpression of *HER2* is observed between 20-29.7% of canine malignant mammary tumors (HSU et al., 2009), overlapping the increase of *HER2* expression exhibited by breast cancer in women (IQBAL; IQBAL, 2014).

Several of these similarities underscore dogs as an excellent model to study novel biological patterns and therapeutic targets in diverse types of cancer. Furthermore, the advances in veterinary oncology research has promoted and verified a substantial interest and discoveries of epigenetic alterations that assist in the development of many canine tumors, several of which are also seen in human oncology. In the following section, we highlight these epigenetic alterations and potential drugs aimed at targeting these epigenetic regulators.

2.3 DNA methylation and canine cancer

DNA methylation is probably the most studied epigenetic modification in animals and plants, playing a fundamental role in development, differentiation, and reproduction. DNA methylation occurs when a DNA n-methyltransferase (DNMT) adds a methyl group to cytosine residues in CpG dinucleotides. These CpG dinucleotides are occasionally enriched in some regions of the DNA called to CpG islands (CGIs) which in turn are preferentially located at gene promoters. DNA methylation results in the silencing of gene expression by essentially two different mechanisms: 1) DNA methylation can provide binding sites for methyl-binding domain proteins which in turn can interact with histone deacetylases (HDACs), reducing chromatin accessibility and repressing gene activation; 2) methylation can prevent gene expression by blocking transcription factors to bind to the promoter regions of genes impeding transcription activation (GREENBERG;

BOURC'HIS, 2019). Beyond gene promoters, CpG methylation is also found in repetitive sequences, gene bodies, and intergenic regions, which can influence gene expression of different approaches (JONES, 2012). CpG hypermethylation in gene bodies, for example, is associated with increased gene expression (YANG et al., 2014).

Under normal conditions, most CpG sites in the genome are methylated while the CGIs are usually unmethylated. In contrast, cancer cells exhibit a genome-wide hypomethylation and CGIs promoter hypermethylation (EHRLICH, 2002). Genome-wide hypomethylation usually occurs in genomic regions including repetitive sequences, retrotransposons and CpG poor promoters resulting in chromosomal rearrangement, activation, and translocation of retrotransposons and, consequently, induce genomic instability. Besides, loss of methylation may lead to activation of proto-oncogenes, such as *RAS*, *S-100* and *MAGE* (EHRLICH, 2009). Genomic hypomethylation has likewise been observed in canine leukemia and lymphoma. In canine leukemia and lymphoma cases, 30 and 69% respectively were found to be genome-wide hypomethylated. Furthermore, these unusual methylation patterns are associated with the early phases of tumor transformation and progression in canine leukemia and lymphoma (PELHAM; IRWIN; KAY, 2003), just as has been observed in different types of human cancer (NARAYAN et al., 1998; JACKSON et al., 2004; ALVAREZ et al., 2011). These findings were the first to report global hypomethylation in canine cancer and consequently, to show similarities between the epigenetic landscape in canine and human cancer.

Also, other canine cancer types display global hypomethylation. A recent study has shown that genome-wide hypomethylation was frequently found in grade III canine mast cell tumor, which is the most common skin tumor in dogs, thus correlating DNA hypomethylation with the aggressiveness of this type of cancer (MORIMOTO et al., 2017). In addition, dogs bearing non-Hodgkin lymphoma (NHL) exhibit higher DNA global hypomethylation of circulating leukocytes in comparison with healthy dogs (EPIPHANIO et al., 2019). DNA hypomethylation was also observed in canine lung cancer samples and in metastatic osteosarcoma from the primary lung cancer (HERRERA et al., 2015). Thus, albeit a still low number of reports, genome-wide hypomethylation seems to be a common feature of at least some types of canine cancer.

Hypermethylation of CGIs also contributes to the development and promotion of cancer through the silencing of tumor suppressor genes. In human cancer, many tumor suppressor genes such as *Rb*, *p16*, *RASSF1*, *CDH1*, *TIMP3*, and *BRCA1* have been shown to possess hypermethylated promoter regions (CHEUNG et al., 2009). These genes are associated with processes including cell cycle, apoptosis, metastasis and DNA repair and, consequently, silencing might induce cancer. Until 2008, no article has shown the presence of promoter hypermethylation in dogs. The first report showing hypermethylation of a tumor suppressor gene in canine cancer was in canine NHL, describing the profile of *DLC1* gene methylation in this cancer. *DLC1* is a tumor suppressor gene found to be highly methylated in human NHL (SHI et al., 2007). Bryan and others performed Methylation Specific PCR (MSP) and combined bisulfite restriction analysis (COBRA) to demonstrate the presence of DNA methylated in *DLC1* in six of 13 canine NHL samples and two of 3 canine chronic lymphocytic leukemia, providing, for the first time, information regarding hypermethylation in canine cancer (BRYAN et al., 2008). In addition, there was an association between *DLC1* hypermethylation and the malignant phenotype of NHL. However, hypermethylation of the *DLC1* promoter was not associated with silencing of *DLC1* expression and did not correlate with survival (BRYAN et al., 2009). Hypermethylation of *TNF-α* has been shown in human and canine melanoma cells by performing MSP. In addition, it has been observed that the methylation status and the level of *TNF-α* expression were inversely correlated in canine melanoma cell lines and melanoma tissues (NOGUCHI et al., 2015a). Both, human and canine melanoma cells have hypermethylated DNA in the CpG islands of the microRNA-203 (NOGUCHI et al., 2015b), a suppressor of growth in melanoma cells, as shown by bisulfite sequencing and MSP (NOGUCHI et al., 2012a). Hypermethylation and epigenetic silencing has also be observed for several other important tumor suppressor genes such as Tissue factor pathway inhibitor 2 (*TFPI-2*) (FERRARESSO et al., 2014), death-associated protein kinase (*DAPK*) (SATO et al., 2014), cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*), *HOXD10*, *FGFR2*, *ITIH5*, and *RASAL3* in B-cell lymphoma (FUJIWARA-IGARASHI et al., 2014; FERRARESSO et al., 2017; ROS et al., 2018). In addition, *DAPK* hypermethylation is associated with overall survival and considered a negative prognostic factor in canine high-grade B-cell Lymphoma (SATO et al., 2018). In canine acute myeloid leukemia, a heterogeneous pattern of DNA methylation was observed with subsets of cases hypermethylated or hypomethylated when compared with

healthy tissues (BRONZINI et al., 2017). Genome-wide analysis of DNA methylation in canine lymphomas revealed that lymphoma cells have gained methylation at CpG sites located in CGIs that were unmethylated in normal peripheral blood mononuclear cells (PBMCs), used as controls. In contrast, CpG sites outside CGIs lose methylation in lymphoma cells compared to the healthy PBMCs (YAMAZAKI et al., 2018).

Some evidence points to downregulation of *BRCA1*, an important tumor suppressor in mammary cancer, in canine mammary samples (IM et al., 2013; QIU et al., 2015). However, the mechanism responsible for the decrease of *BRCA1* expression is not well understood. Recently, a study showed *BRCA1* hypermethylation in canine mammary tumors. However, the rate of *BRCA1*-hypermethylated samples was very low (1/15 – 6.7%) making it difficult to conclude that *BRCA1* downregulation is a consequence of *BRCA1* promoter hypermethylation (QIU; LIN, 2016). Attempts to studying both DNA methylation and histone modifications in canine cancer have been performed. Canine lymphoid tumor cell lines with different drug-sensibility were analyzed using bisulfite sequencing and chromatin immunoprecipitation and it was found that DNA methylation and histone H3 acetylation are involved in *ABCB1* gene expression (TOMIYASU et al., 2014a). ABCB1 is a P-glycoprotein highly expressed in several different types of human cancer and has been recognized to be a key player in the multidrug resistance phenotype (ROBEY et al., 2018). Another study observed that CGIs of the *ABCB1* gene were hypomethylated in dogs with lymphoma. However, the authors did not find a correlation between the methylation status and levels of *ABCB1* mRNA expression in these samples (TOMIYASU et al., 2014b). Both human and canine mammary carcinomas can present deregulation of Estrogen receptor α (*ERα*) and its expression levels guide the prognosis and kind of therapy for the respective patient. In human breast cancer, the most aggressive type of cancer is triple-negative breast cancer, featured by the lack of *ERα* expression, which is mainly attributed to *ERα* promoter methylation (OTTAVIANO et al., 1994; PRABHU et al., 2012). However, no significant variation in methylation patterns were found between *ERα*-positive canine mammary carcinomas and *ERα*-negative canine mammary carcinomas pointing to a difference of *ERα* regulation mechanisms between human and dogs (BRANDÃO et al., 2018).

TET proteins are responsible to catalyze the successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and

5-carboxylcytosine (5caC), promoting DNA demethylation. *TET2* is considered an important tumor suppressor gene being commonly mutated in hematopoietic tumors but rarely in solid tumors (RASMUSSEN; HELIN, 2016). In hematopoietic canine tumors, *TET2* mutations have also been observed in canine mast cell tumors but in very low frequency (2.7%) (ZORZAN et al., 2015), contrary the *TET2* mutations rate in human systemic mastocytosis, which are observed in 40% of the cases (TEFFERI et al., 2009). *TET2* was also found mutated in canine T-cell lymphoma samples, but in low frequency as well (MCDONALD et al., 2018). All this information regarding DNA methylation and Canine Cancer are summarizing in **Supplementary Table 1, Appendix A**.

2.4 Histone modifications and canine cancer

In the nucleus, DNA is compacted and complexed by proteins called histones resulting in a DNA-protein complex named chromatin. Histone proteins can be divided in two groups: core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5). Together, these proteins make up the nucleosome (the unit of chromatin), which is 'coated' with 146 base pairs of DNA. Histones contain a C-terminal domain and an unstructured N-terminal domain, commonly referred to as histone tail (LUGER et al., 1997). These histone tails are susceptible to different post-translational covalent modifications such as methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, deamination, propionylation, and butyrylation (BANNISTER; KOUZARIDES, 2011; KEBEDE; SCHNEIDER; DAUJAT, 2015) which directly affect the chromatin structure by recruiting enzymes able to remodel chromatin. Consequently, histone modifications are tightly linked to cellular processes including replication, repair, and recombination (KOUZARIDES, 2007). Furthermore, because of their influence on regulating the accessibility of chromatin to the transcriptional machinery, some modifications are responsible to regulate gene expression (LAWRENCE; DAUJAT; SCHNEIDER, 2016). For instance, histone acetylation neutralizes the positive charge of histone lysines and consequently results in loosening the DNA packing around histones making it more accessible to transcriptional activation (Hebbes et al. 1988). On the other hand, histone methylation is usually associated with both transcriptional activation and repressions, depending on which residue is modified or the degree of methylation (mono-di or thimethylation).

Just as aberrations in DNA methylation patterns can lead to cancer development, altered histone modifications and chromatin changes can be observed in cancer cells. For example, some studies revealed a global loss of acetylated H4-lysine 16 (H4K16ac) and H4-lysine 20 trimethylation (H4K20me3) in different cancer cell lines and primary tumors, such as leukemia, breast, lung, and colon cancer (FRAGA et al., 2005). In addition, alterations of H3K9me and H3K27me patterns are also observed in different types of human cancer, including bladder, colorectal, glioma, breast, and lung cancer (NGUYEN et al., 2002; MICHALAK; VISVADER, 2016; NICHOL et al., 2016; ROWBOTHAM et al., 2018; WAN; LIU; CHAN, 2018). Accordingly, the expression of enzymes responsible for these modifications, histone acetylases/deacetylases (HATs and HDACs) and histone methyltransferases (HMTs) are observed to be dysregulated in cancer. HDACs are often overexpressed in human cancer (GLOZAK; SETO, 2007) but HATs can be also altered (IYER; ÖZDAG; CALDAS, 2004). HMTs such as EZH2 and G9a have be found overexpressed in different types of cancer (CASCIELLO et al., 2015; KIM; ROBERTS, 2016; TU et al., 2018).

Some studies have shown dysregulation of histone modifications exhibiting key roles in the development and progression of canine cancer (**Supplementary Table 2, Appendix A**). Recently, in canine urothelial carcinomas samples, significant deacetylation of histones compared to normal samples was observed and these lower acetylation levels were associated with a poor prognosis of the animals (ETO et al., 2019). *SETD2* gene, a histone methyltransferase and an important tumor-suppressor, was found to be mutated in 21% of canine osteosarcoma samples and showed a variety of mutation types including frameshift, nonsense, splice, and missense mutations (SAKTHIKUMAR et al., 2018). Another study detects *SETD2* somatic point mutations, deletions and chromosomal translocations in 42% of canine osteosarcoma samples (GARDNER et al., 2019). Just like in human cancers, overexpression of *EZH2* was found in canine lymphoma, melanoma, basal cell tumors, squamous cells carcinoma, prostate and mammary cancer (CHOI et al., 2016, 2018).

Along with the simple carcinomas, complex carcinomas present the most common type of canine mammary cancer and are characterized by the presence of epithelial and myoepithelial cells (GOLDSCHMIDT et al., 2011). These types of mammary cancers seem to be profoundly influenced by the epigenetic landscape and recent findings suggest

they originate from epigenomic rather than genomic alterations. Analysis of whole-genome sequencing, whole-exome sequencing, RNA-seq, and/or high-density arrays on twelve canine mammary cancer cases, including seven simple carcinomas and four complex carcinomas showed that, contrary to simple carcinomas, complex carcinomas did not have any copy number abnormalities and also low mutation rates. Conversely, complex canine mammary carcinomas displayed a number of epigenetic dysregulations, such as downregulation of 35 chromatin-modification genes or abnormally enriched activating histone modification H4-acetylation, while showing a reduction in the repressive histone modification H3K9me3 (LIU et al., 2014).

2.5 Non-coding RNAs and canine cancer

Non-coding RNAs (ncRNAs) are defined as RNA molecules that are not translated into a protein and, for a long time, their functions in the genome were not well understood. However, with the recent advances in cell biology, transcriptomic and bioinformatic tools, it has become possible to elucidate the role of these molecules regulating biological pathways and processes. These ncRNAs are classified as microRNAs (miRNAs), transfer RNAs (tRNAs), PIWI-interacting RNAs (piRNAs), long non-coding RNAs (lncRNAs), pseudogenes and, circular RNAs (circRNAs) (SLACK; CHINNAIYAN, 2019). In this review, miRNAs and lncRNAs are addressed (**Supplementary Table 3, Appendix A**).

MicroRNAs (miRNAs) are a class of non-coding RNAs encoded in the genome. The first miRNA description was in the nematode *C. elegans* (Lee et al, 1993). Since then more than 15.000 miRNAs have been identified (www.mirbase.org). Several miRNAs are expressed in many different species and are highly conserved amongst them. These molecules were found to develop a key role in many biological processes including cell proliferation, metabolism, development, differentiation, apoptosis and stress response (MISKA, 2005; KLOOSTERMAN; PLASTERK, 2006; ALBERTI; COCHELLA, 2017). They control these processes by regulating gene expression through post-transcriptional mechanisms. MiRNAs bind to their target mRNA and downregulate it via one of two different mechanisms: 1) when miRNA and mRNA have a full complementarity, the miRNA triggers the degradation of the target mRNA; 2) miRNAs can also bind to the mRNA 3'UTR regions with incomplete complementarity, leading to translational repression (WILCZYNSKA; BUSHELL, 2015) (**Figure 2**).

Several lines of evidence have shown that miRNAs play an important role in the development of diseases in humans including cardiovascular diseases (ZHOU et al., 2018), neurodegenerative diseases (MACIOTTA; MEREGALLI; TORRENTE, 2013) and several types of cancer. The mechanisms responsible for miRNA dysregulation in human cancer has been well elucidated and include amplification or deletion of miRNA genes, aberrant transcriptional control of miRNAs due to the dysregulation of some transcription factors such as *C-Myc* and *P53*, dysregulated epigenetic changes with some studies showing aberrant patterns of DNA methylation and histone acetylation in miRNA genes, and defects in miRNA biogenesis machinery (CALIN et al., 2002; O'DONNELL et al., 2005; THOMSON et al., 2006; HAN et al., 2007; ILIOU et al., 2014; PENG; CROCE, 2016).

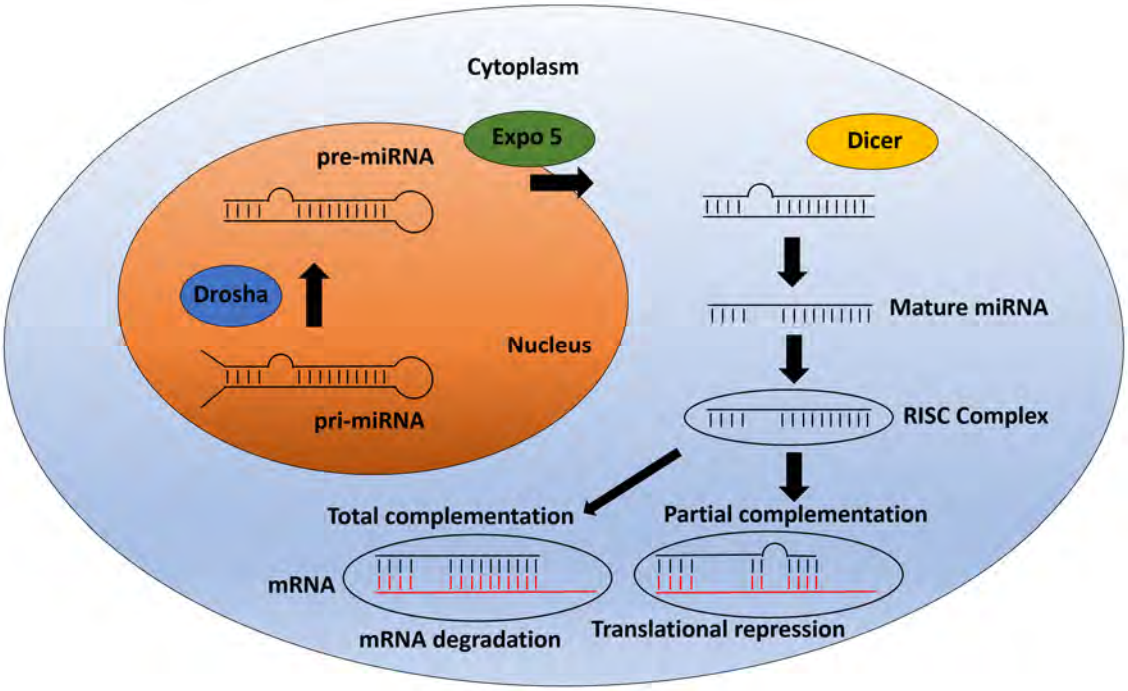


Figure 2. miRNA pathway: from biogenesis to mRNA inhibition. After pri-miRNA generation by transcription, the microprocessor complex Drosha processes and cleaves the pri-miRNA to produce the precursor-miRNA (pre-miRNA). Then, the pre-miRNA is transported from the nucleus to cytoplasm by Exportin 5. In the cytoplasm, pre-miRNA is processed by Dicer to produce the mature miRNA. The mature miRNA is incorporated into a protein complex termed RISC. Finally, this complex induces gene inhibition in two different ways. 1) The mRNA can be degraded if there is total complementation between the miRNA and the mRNA. 2) In the case of partial complementation, there is a translational repression.

The first evidence of miRNA dysregulation in canine cancer was described for canine mammary cancer. Both, *miR-29* and *miR-29b* were found to be upregulated in

canine mammary cancer samples. Furthermore, the same study showed that *miR-15a* and *miR-16* are significantly downregulated in canine ductal carcinomas while *miR-181b, -21, -29b*, and *let-7f* showed a significant upregulation in canine tubular papillary carcinomas (BOGGS et al., 2008). Thenceforth, some studies have described miRNA profiling in different types of canine cancer such as mast cell tumors (FENGER et al., 2014), osteosarcoma (FENGER et al., 2016), hemangiosarcoma (GRIMES et al., 2016), prostate cancer (KOBAYASHI et al., 2017), canine multicentric lymphoma (CRAIG et al., 2019), and melanoma (RAHMAN et al., 2019; USHIO et al., 2019). Interestingly, important and well-described miRNAs in human cancer including *miR-9*, *miR-18a, miR-126, miR-383, and miR-204* were found to be dysregulated in canine cancer. The presence of *MiR-181* and *miR-17-5p* were observed in B- and T-cell lymphomas compared to non-neoplastic cells (MORTARINO et al., 2010). Furthermore, miRNA dysregulation appears to have a key role in regulating metastasis in some canine cancer. The expression of 14 miRNAs were significantly different between metastasizing and non-metastasizing uveal melanomas, highlighting *cfa-miR-362, cfa-miR-155, cfa-miR-182*, and *cfa-miR-124* as strongly associated with the metastasizing class in this type of cancer (STARKEY et al., 2018). Ten miRNA (*cfa-let-7c, cfa-miR-10b, cfa-miR-26a, cfa-miR-26b, cfa-miR-29c, cfa-miR-30a, cfamiR-30b, cfa-miR-30c, cfa-miR-148a* and *cfa-miR-299*) were validated and showed significant different expression in metastatic and non-metastatic mammary tumors (BULKOWSKA et al., 2017). *MiR-9* was found to be overexpressed and associated with metastasis in mast cells tumors and osteosarcoma (FENGER et al., 2014, 2016), while *miR-34a* also appeared to be associated with invasion ability in canine osteosarcoma cell lines (LOPEZ et al., 2018). Some miRNAs also correlated with tumor grading in canine splenic lymphoma (ALBONICO et al., 2013).

Circulating miRNAs detected on liquid biopsies such as blood and urine may provide diagnostic and prognostic information regarding cancer (CUI et al., 2019). *MiRNA-214* and *-126* have been considered potential diagnostic and prognostic biomarkers for canine neoplastic diseases. In a recent study, using 181 cases of canine neoplastic diseases and healthy controls, circulating *miRNA-214* was considered a good diagnostic marker in sarcomas, whereas levels of the circulating *miRNA-126* was high in most of the types of canine tumors (HEISHIMA et al., 2017). These same miRNAs were demonstrated to have a strong potential to predict the outcome of canine appendicular osteosarcoma patients receiving amputation and chemotherapy (HEISHIMA et al., 2019).

Dogs with disseminated histiocytic sarcoma and carcinomas showed downregulation of circulating *Let-7g* (BØRRESEN et al., 2017). Another study described the profile of circulating serum miRNAs in dogs with lymphoma. Four miRNAs (*let-7b, miR-223, miR-25, miR-92a*) were significantly reduced in dogs with lymphoma, whereas *miR-423a* levels were significantly increased compared to the controls (FUJIWARA-IGARASHI et al., 2015). *MiR-99a* was also differentially expressed in the plasma of dogs with lymphoma (CRAIG et al., 2019). Circulating miRNAs detected in the urine are also been detected in canine cancers. In canine bladder transitional cell carcinomas, *miR-103b* and *miR-16* were considered as potential diagnostic urine biomarkers (KENT et al., 2017). Analysis of the miRNA profiles within the exosomes released from canine tumors has also been made. A recent study observed that canine mammary epithelial cancer cells shed exosomes that contained differentially expressed miRNAs in comparison with normal cells (FISH et al., 2018). In a study performed in canine lymphoma, three miRNAs (*miR-151, miR-8908a-3p, and miR-486*) derived from exosomes demonstrated to be differently expressed between vincristine-sensitive and resistant lymphoma cell lines supporting a role of these miRNAs in the resistance of this cancer (ASADA et al., 2019).

LncRNAs are non-coding transcripts greater than 200 bp in length and some studies demonstrated the influence of these molecules in gene expression at the epigenetic, transcriptional, and post-transcriptional levels. One of the most classical mechanisms through which lncRNAs regulate gene expression is by association with chromatin modeling complexes and transcription factors, influencing transcriptional repression and activation of gene promoters. For example, the well-characterized lncRNA *HOTAIR* can bind to epigenetic complexes such as PRC2 and LSD1/CoREST/REST, modulating histone methylation (TSAI et al., 2010). In addition, lncRNAs binds directly to DNA, mRNAs, and/or miRNAs affecting and regulating their respective functions and levels (MONDAL et al., 2015; DU et al., 2016; ZHANG et al., 2018).

The lncRNAs play a fundamental role in the development and physiology of the human organism but can be also associated with disease evolution, especially cancer. *HOTAIR* overexpression, for example, has been associated with an increase of metastasis, invasiveness, and, consequently, to poor outcomes in breast and other types of cancer (GUPTA et al., 2010; LI et al., 2016). Many oncogenic lncRNAs including *THOR* (HOSONO et al., 2017), *ARLNC1* (ZHANG et al., 2018), *SAMSOON* (LEUCCI et al.,

2016), and *EPIC1* have also been associated with different types of cancer such as lung, prostate, melanomas, ovarian, and pancreatic cancer (WANG et al., 2018).

Due to the importance of lncRNAs in the genome and their association with different human diseases, the canine lncRNA profile has also been described (HOEPPNER et al., 2014). An alignment-free program that accurately annotates lncRNAs, FEELnc, was used on a real data set of 20 RNA-Seq data from 16 different canine tissues produced by the European LUPA consortium to expand the canine genome annotation including 10.374 novel lncRNAs and 58.640 mRNAs transcripts (WUCHER et al., 2017). This study particularly highlighted duplications of lncRNAs in dog. Interestingly, among the novel lncRNAs genes, around 15% were also described as non-protein coding genes in the human GENCODE. Finally, with this set of data was possible to annotate three new cancer susceptibility candidate lncRNAs in dogs, which are well described in human cancer, including *CASC9*, associated with esophageal squamous cell carcinoma (HAO et al., 2015), *MALAT1*, associated with metastasis in lung cancer (GUTSCHNER et al., 2013), and *IFNG-AS* that plays an important role in T-cell differentiation (MULJO; ZHU; ZHAO, 2013). Another study, observed more than 900 dog-human conserved lncRNAs using comparative genomics. The authors confirmed the annotation of well-studied lncRNAs in dogs, such as *HOTAIR*, *MALAT*, *NEAT_1*, *PCA3*, *CASC15*, *CASC17*, *CASC18*, *CASC20*, and *INHBA-AS1*. In addition, 44% of the canine lncRNAs are expressed in a tissue-specific manner, which is also widely seen in humans (VERMA et al., 2015). Finally, co-expression analysis suggested that these lncRNAs function as regulatory elements in the dog genome (LE BÉGUEC et al., 2018). Despite the increase of the lncRNA number and description, few lncRNA are functionally and experimentally characterized in dogs, and only few of them have been found to be associated with diseases. For instance, the lncRNA *GDNF-AS* was observed to be involved in a Hereditary Sensory Autonomic Neuropathy (HSAN) in hunting dogs (PLASSAIS et al., 2016).

LncRNAs is also associated with some types of canine cancer. Cross-species analysis of lncRNAs demonstrated that a non-negligible fraction of lncRNA associated with human diffuse large B-cell lymphoma (DLBLC) is also expressed in canine lymphoma (VERMA et al., 2015). A recent study has also developed a methodology to identify lncRNAs in canine DLBCL. The authors concluded that this methodology was

able to quantify the expression of novel and annotated lncRNAs and, interestingly, subclassified the DLBCL in two main groups. Furthermore, these two DLBCL groups showed statistically different survival rates, pointing to the potential of using lncRNAs as prognostic markers using this methodology (CASCIONE et al., 2019). In canine oral melanomas, 417 differentially expressed lncRNAs were identified in comparison with control samples, using deep transcriptome sequencing. Most of these lncRNAs have not yet been functionally characterized, however, lncRNA *ZEB2-AS*, a lncRNA involved in the regulation of the transcription factor *ZEB2* during epithelial-mesenchymal transition (EMT) in human colon, pancreatic, and breast cancer cell lines (BELTRAN et al., 2008), was highly expressed in canine oral melanomas compared to control samples. Other examples of lncRNAs dysregulated in these tumors that are well described in human cancer, were *SOX21-AS1* (YANG et al., 2016), and *CASC15* (LESSARD et al., 2015). Finally, using co-expression network analysis (WGCNA), the differentially expressed lncRNAs were associated with Gene Ontology (GO) biological process including cancer-related genes, cell cycle, cellular response to stress, DNA metabolic process, and carbohydrate metabolism (HITTE et al., 2019).

2.6 Epigenetic drugs to treat canine cancer

Contrary to genetic mutations, epigenetic changes occurring in cancer are potentially reversible. There is thus the possibility of treating cancer with epigenetic drugs and, consequently, reverse some malignant phenotypes including metastasis potential (HENDRIX et al., 2007; BOCKHORN et al., 2014), tumorigenicity (FLAVAHAN; GASKELL; BERNSTEIN, 2017) and multidrug resistance (BROWN et al., 2014). Several efforts have been undertaken for the development of epigenetic drugs targeting defective DNMTs and histone modifying enzymes as well as reader domains in cancer, but, unlike in human oncology, epigenetic drugs are still little in use in veterinary oncology, as we outlined below. However, it is important to emphasize that dogs are used as models in most preclinical tests for these drugs providing an overview of the possible side effects of these anticancer agents in this species.

2.6.1 DNA methyltransferases inhibitors

The first two epigenetic cancer drugs, the 5-azacytidine (5-azaC or azacitidine) (TKACZYNSKI; ŠMEJKAL; ŠORM, 1964) and the 5-aza-2′-deoxycytidine (5-aza-dC or decitabine) (CEIFJAOEKCA et al., 1964) were synthesized in 1964 but only approved

by the FDA in 2004 and 2006, respectively (KAMINSKAS et al., 2005; SABA, 2007) . Both are DNMT inhibitors and are currently first-line therapy for myelodysplastic syndrome (MDS), a bone marrow disorder that can progress to acute myeloid leukemia (AML). Furthermore, 5-azaC and 5-aza-dC are administrated to treat hematological malignancies including chronic myelomonocytic leukemia (CMML) and AML in elderly patients ineligible for intensive chemotherapy (FENAUX et al., 2010; OSSENKOPPELE; LOWENBERG, 2015; DIESCH et al., 2016). Although efficient, this first generation of DNMT inhibitors presents some issues including lack of specificity, which could trigger some side effects, poor bioavailability and limited half-life (GANESAN et al., 2019). Thus, second-generation DNMT inhibitors have been developed including zebularine (YOO; CHENG; JONES, 2005), CP-4200 (SINGH; CAPALASH, 2013) and guadecitabine (KANTARJIAN et al., 2017). For solid tumors, azanucleoside-based therapies are also being tested in phase I/II clinical trials in several types of cancer (FAN et al., 2014; NERVI; DE MARINIS; CODACCI-PISANELLI, 2015).

Due to their promising results in human cancer, DNMT inhibitors have been tested, although at a low scale, in canine cancer (**Supplementary Table 4, Appendix A**). The first 5-aza-dC test report in dogs was published in 1983. Dogs were used to investigate the plasma and cerebrospinal fluid pharmacokinetics of 5-aza-dC and the results showed that the compound could be rapidly cleared from plasma and cross the blood-CSF barrier resulting in potentially and cytotoxic concentrations by infusion (CHABOT, 1983). However, in this study, dogs were only used as experimental models, not aiming treatment of canine cancer. Dogs with naturally occurring invasive urothelial carcinoma were treated with subcutaneous 5-aza-C. Of the 18 dogs in the study, partial remission was achieved in 22%; 50% showed stable disease, whereas in 22% the cancer progressed. The subcutaneous 5-azaC strategy in dogs was considered promising and important for the translation and design of human urothelial carcinoma clinical trials (HAHN et al., 2012).

In human and canine melanoma cells, a recent study has shown a new apoptosis-inducing mechanism of 5-aza-dC through demethylation and induction of cytotoxic cytokines such as TNF-α in *in vitro* and *in vivo* experiments, suggesting a potential therapeutic agent for human and canine melanomas (NOGUCHI et al., 2015a). 5-azaC

reduced *in vitro* growth, invasion, tumorigenicity, mitochondrial activity and increased the susceptibility to apoptosis of breast cancer cells from human, canine and feline species. In addition, 5-azaC was toxic to mammary cancer cells but not to healthy mammary cells lines from these species, indicating a therapeutic window and sustaining animals as useful models for pre-clinical evaluation of new drugs targeting breast cancer (HARMAN et al., 2016). Likewise, second-generation of DNMT inhibitors have been tested in canine models. Zebularine was able to inhibit DNMT1 and promote global demethylation of canine malignant lymphoid cells resulting in dose-dependent apoptosis (FLESNER; KUMAR; BRYAN, 2014). Toxicological and pharmacokinetic studies with Zebularine were performed using laboratory animals and dogs with natural occurring tumors. Plasma zebularine clearance was constant. Laboratory dogs treated with a daily oral zebularine dose of 4 mgkg-1 developed some side effects including neutropenia, found in all dogs, thrombocytopenia in one dog, anorexia in 4 dogs and dermatological changes. In the dogs with tumors, thrombocytopenia was observed in one dog. No other hematologic abnormalities, serum biochemical abnormalities or dermatologic changes were detected. Despite important information regarding plasma pharmacokinetics and toxicity of zebularine in dogs, more studies should still be performed to observe the anticancer activity of zebularine in this specie (FULKERSON et al., 2017).

2.6.2 Histone deacetylase inhibitors (HDACi)

Since HDACs are often dysregulated in different types of cancer, many efforts have been made to develop efficient HDAC inhibitors. In human multiple myeloma, ovarian, gastric, breast, pancreatic, and prostate cancer, for example, HDACs are overexpressed and associated with poor outcome (HALKIDOU et al., 2004; KIM et al., 2004; ZHANG et al., 2005; WEICHERT et al., 2008a, 2008b; MITHRAPRABHU et al., 2014; DAMASKOS et al., 2017; LI et al., 2017). Different patterns of HDAC1 have been established as prognostic marker in osteosarcoma. Whereas in primary osteosarcoma, cells showed a high expression of HDAC1 and 2, low levels of HDAC1 were associated with the presence of initial metastasis (CHAIYAWAT et al., 2018). Considering the relevance of this target class, four HDACs inhibitors have been approved for cancer treatment by the U.S Food and Drug Administration (FDA): vorinostat, romidepsin, belinostat, and panobinostat. The first HDACi, SAHA (Vorinostat, Zolinza™, Merck & Co, Inc., USA), was approved by FDA in 2006 and since then HDACi are being

developed for the treatment of T-cell lymphoma (MANN et al., 2007; GRANT et al., 2010; LEE et al., 2015) and multiple myeloma (RAEDLER; WRITER, 2016). Furthermore, other studies and clinical trials showed the effects of Vorinostat in hematological and solid cancers including pancreatic (KUMAGAI et al., 2007), ovarian (MA et al., 2017), prostate (KAUSHIK et al., 2015), and breast cancer (LUU et al., 2018).

HDACis have been also shown good effects in canine cancer (**Supplementary Table 5, Appendix A**). Vorinostat reduced the viability and increased apoptosis in a dose-dependent manner besides decreasing phosphorylation in oncogenic pathways including Akt-Ser^{473} and mTOR in canine osteosarcoma cell lines (MURAHARI et al., 2017). In canine urothelial carcinoma cells, Vorinostat inhibited the growth and induced G0/G1 cell cycle arrest through the upregulation of p21 and dephosphorylation of Rb in these cancer cells (ETO et al., 2019). Both studies showed that Vorinostat was able to induce histone H3 acetylation in these canine cancer cells. The effects of another HDACi, sulforaphane, has been shown on canine osteosarcoma cells, significantly decreasing cell invasion and downregulating focal adhesion kinase (FAK) signaling (RIZZO; LEVINE; WAKSHLAG, 2017).

A panel of seven HDACis were tested, in a well-established canine B-cell Lymphoma cell line, CLBL-1 using *in vitro* and *in vivo* (xenograft) models. All HDACis tested exhibited dose-dependent inhibitory effects on the proliferation of CLBL-1 cells. Furthermore, Panobinostat, the most potent HDACi tested *in vitro*, inhibited CLBL-1 xenograft tumor growth, triggering acetylation of H3 and apoptosis in *vivo* (DIAS et al., 2018a). Panobinostat also efficiently inhibited the growth of tumors in xenograft models inoculated with a modified and bioluminescent canine B-cell lymphoma cell line (DIAS et al., 2018b). Trichostatin A (TSA), an antifugical agent with properties to selectively inhibit histone deacetylase activity in mammalian cells has shown inhibitory effects of proliferation and apoptosis in cancer cells (VIGUSHIN et al., 2001). *In vitro* inhibitory effects of TSA were also shown on canine grade 3 mast cell tumor, decreasing cell viability, by increasing apoptosis and the number of cells in sub-G1 phase of cell cycle, indicating cell death (NAGAMINE et al., 2011). TSA also inhibited the proliferation of one canine mammary cancer cell line (WATANABE et al., 2009). A novel HDAC inhibitor AR-42, recently in phase I/Ib trials for multiple myeloma and T- and B-cell lymphomas (SBOROV et al., 2017), has shown effects in canine osteosarcoma, prostate,

and malignant mast cancer cells. Cell viability inhibition and induction of apoptosis via activation of the intrinsic mitochondrial pathway were observed in canine osteosarcoma cells treated with AR-42. In addition, AR-42 showed synergistic effects when combined with doxorubicin (MURAHARI et al., 2017). In canine prostate cancer, AR-42 inhibited *in vitro* proliferation in a time- and dose-dependent manner and decreased migration and the incidence of bone metastasis in xenograft models (ELSHAFAE et al., 2017). AR-42 treatment of canine malignant mast cells induced proliferation inhibition, cell cycle arrest, apoptosis, and activation of caspases-3/7. Downregulation of KIT, a commonly mutated gene in malignant mast cells, via inhibition of KIT transcription was also observed. Finally, AR-42 treatment downregulated several important cancer molecules including p-AKT, total AKT, phosphorylated STAT3/5, and total STAT3/5 (LIN et al., 2010).

The effects of HDACs inhibition in combination with other therapies has also been studied in canine cancer. A phase I pharmacokinetic and pharmacodynamic study of combined valproic acid (VPA) and doxorubicin was performed in spontaneous canine cancers. Of the 21 dogs treated in this study, two presented complete responses (10%) (both lymphomas), three presented partial responses (14%) (lymphoma, melanoma and lung carcinoma), five showed stable disease after treatment (24%) (osteosarcoma, renal cell carcinoma, apocrine gland adenocarcinoma, melanoma, soft-tissue sarcoma) and eleven exhibited progressive disease (58%) (WITTENBURG; GUSTAFSON; THAMM, 2010). In another study from the same group, using both human and canine osteosarcoma (OS) cells, pre-incubation with VPA followed by doxorubicin increased the growth inhibition and apoptosis rates in both human and canine OS cells, associated with a dose-dependent increase in nuclear doxorubicin accumulation, supporting a potential addition of HDACis for treatments of human and canine OS (WITTENBURG et al., 2011).

2.6.3 Alternative Epigenetic Targets

Due to successful development of DNMT and HDAC inhibitors and the use of these molecules in the treatment of diseases, new classes of epigenetic drugs has been developed to target epigenetic writers, erasers and even epigenetic readers. Several examples are reported including histone methyltransferases inhibitors such as DOT1L (STEIN et al., 2018), EZH2 (GULATI; BÉGUELIN; GIULINO-ROTH, 2018), and G9A inhibitors (CHARLES et al., 2019). Many studies are also observing the effects of lysine demethylases inhibition including LSD1 and LSD2 (FANG; LIAO; YU, 2019) and

epigenetic readers such as bromodomains with the BET (Bromo and Extra terminal) family comprising BRD2, BRD3, BRD4, and BRDT as the most prominent targets for drug discovery (DOROSHOW; EDER; LORUSSO, 2017). During the past years, the Structural Genomic Consortium (SGC), a public-private partnership that supports the discovery of new medicines through open access research, has designed and developed a set of tool compounds for epigenetic targets with clearly defined properties (MÜLLER; BROWN, 2012; BROWN; MÜLLER, 2015).

Our group recently screened a small-molecule library containing 27 of these developed epigenetic inhibitors in canine mammary cancer cell lines (CMCs). We observed three inhibitors inducing significant reduction of cell viability in CMCs including (+)-JQ1 (BET family inhibitor), NVS-CECR2-1 (CECR2 inhibitor), and UNC1999 (EZH2/1 inhibitor). Furthermore, BET inhibition by (+)-JQ1 was very efficient to inhibit CMCs colony and tumorsphere formation, demonstrating an effect on tumorigenicity and self-renewal (**Figure 3**) (XAVIER et al., 2019) . Inhibition of SET methyltransferase by shRNA and FTY720, reported to directly interact with SET proteins, suppressed cell proliferation, colony formation, and *in vivo* tumor growth of canine mammary and osteosarcoma cell lines. Furthermore, SET knockdown repressed mTOR and NF-kB signaling in both types of canine cancer (KAKE et al., 2017; TSUJI et al., 2019). Using BB-Cl-Amidine (BB-CLA) to inhibit protein-arginine deiminases (PADs) resulted in the decrease of viability and tumorigenicity of canine mammary cancer cells, activating endoplasmic reticulum stress pathway in these cells (LEDET et al., 2018).

Dogs have also been used as models for pre-clinical trials of LSD1 inhibitors. A recent study, showed that the LSD1 inhibitor GSK2879552 caused severe but reversible toxicities in dogs including thrombocytopenia, neutropenia, myelofibrosis, and congestion with and without lymphoid necrosis in lymphoid organs (FANG; LIAO; YU, 2019). However, studies demonstrating the effects of these new alternative epigenetic drugs in dogs are very scarce (**Supplentary Table 6, Appendix A**).

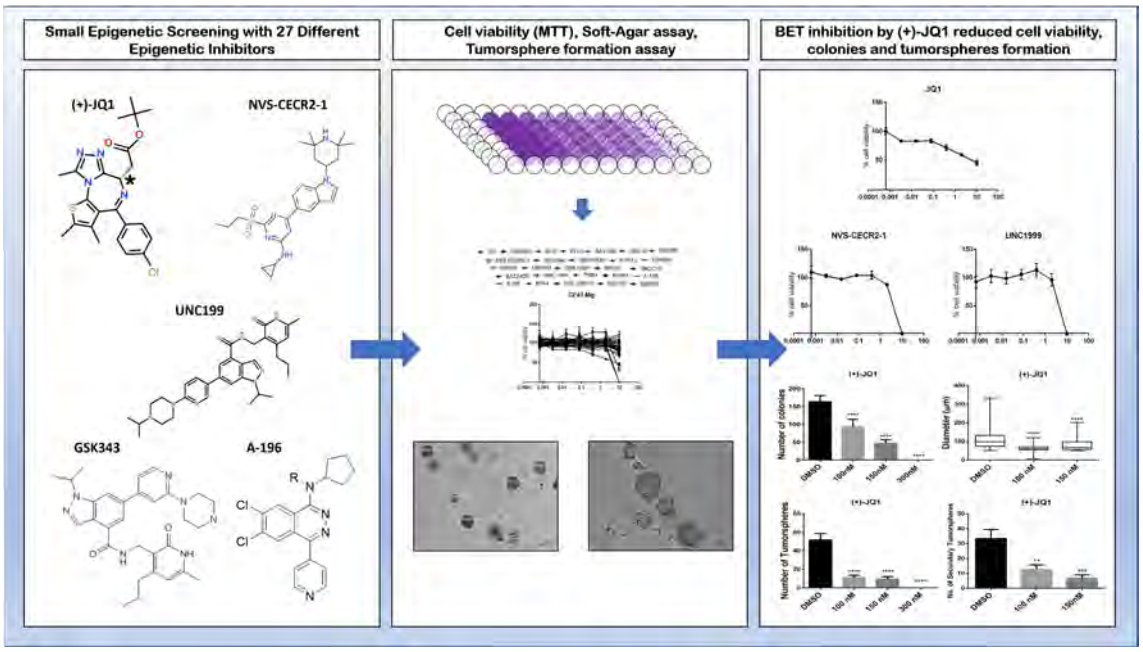


Figure 3. Effects of some alternative epigenetic inhibitors in canine mammary cancer cells. A small library of 27 epigenetic inhibitors was screened in order to determine effects regarding cell viability, tumorigenicity, and self-renewal assessed by 3D cell culture models such as colony formation in soft-agar and tumorspheres formation in low-adherent plates (WEISWALD; BELLET; DANGLES-MARIE, 2015). The (+)-JQ1 (BET family inhibitor), NVS-CECR2-1 (CECR2 inhibitor), and UNC1999 (EZH2/1 inhibitor) decreased cell viability of CF41.Mg canine mammary cancer cell line. Furthermore, (+)-JQ1 exhibits a strong impact on colony and tumorspheres formation, demonstrating effects on tumorigenicity and self-renewal phenotypes (XAVIER et al., 2019).

2.7 BarkBase: a canine epigenomic resource

The abundance of information acquired in recent years on human genomics both in healthy and diseased tissues enabled the construction of powerful platforms of data that can be used for the deep investigation of different phenotypes. Resources such as ENCODE (DUNHAM et al., 2012), GTEx (LONSDALE et al., 2013), Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga) and NIH reference human epigenome (ROADMAP EPIGENOMICS CONSORTIUM et al., 2015), are constantly fueled with large numbers of information generated with next-generation bioinformatic tools and are extremely important for the elucidation of the most diverse diseases that affect humans and their therapeutic advances. With the same purpose, a ~7 gigabytes genomic data platform, the BarkBase resource, has been recently developed (MEGQUIER et al., 2019). BarkBase contains data for 27 adult tissue types, with biological replicates, from five adult dogs, paired with 30x whole genome sequence data. RNA sequencing data are

complemented by whole genomic sequencing and assay data for transposable-accessible chromatin using sequencing (ATAC-seq). All these genomic and epigenomic data from healthy canine tissues will be highly important and useful for future studies in canine cancer providing the basis of a high-quality tool to compare the findings found in canine cancer tissues with healthy tissues. Furthermore, BarkBase introduces a reliable and solid resource to support comparative studies between canine and human species (http://www.barkbase.org/).

2.8 Conclusion and Perspectives

Epigenetic alterations are present and possibly regulating several types of canine cancer. Furthermore, many of these epigenetic alterations in canine cancer are also observed in human cancer including genome-wide hypomethylation, hypermethylation of tumor suppressor genes, aberrant histone modifications, and dysregulation of non-coding RNAs (**Table 1**). These data suggest a potential approach using the canine model to determine new epigenetic mechanisms regulating cancer, diagnostic/prognostic markers and targets for the development of new anticancer drugs. Interestingly, these findings increase the possibility to investigate which environmental factors play a role in epigenetic alterations both in human and dogs, since both species are exposed to the same carcinogens in the environments during their life and, surprisingly few studies aimed to observe the environmental risk factors in canine cancer (HAYES; HOOVER; TARONE, 1981; BUKOWSKI; WARTENBERG; GOLDSCHMIDT, 1998; KELSEY; MOORE; GLICKMAN, 1998; GAVAZZA et al., 2001; ZANINI et al., 2013). Despite these similarities, some differences regarding the epigenetic landscape can be observed in human and canine cancer such as the epigenetic regulation of Estrogen Receptor α (*ERα*) between breast cancer in women and mammary cancer in dogs and the epigenetic regulation of *ABCB1* gene in lymphomas. In addition, most studies aiming to elucidate the epigenetic profile of canine cancer and to determine possible targets and therapies for this disease in these animals are performed using *in vitro* models. Thus, further investigations are needed to confirm the potential of use dogs as a comparative and translational model to study epigenetics.

Currently, therapeutic options to treat canine cancer are basically surgery, radiotherapy, hyperthermia, photodynamic therapy, immunotherapy, and chemotherapy (PAOLONI; KHANNA, 2008). Thus far, there is no epigenetic drug specific to treat

canine cancer or being used in veterinary oncology clinics. This fact is probably a consequence of the lack of solid studies determining the main epigenetic targets in canine cancer. Studies of targeted therapy in dogs using appropriate protocols and models inhibiting epigenetic targets are missing to investigate the potential of epigenetic modulation for the treatment of canine cancer in clinics. Furthermore, all current epigenetic drugs were designed for human treatment. Thus, despite promising *in vitro* results of epigenetic drugs in canine cancer cells, the effect of the compounds was not optimized for canine cancers and side effects may be present due to differences in physiology between man and dog.

Following the exciting development of studying the role of epigenetic reprogramming in human cancer, this area is also emerging in veterinary oncology. Several studies have unveiled epigenetic alterations in canine cancer types and, importantly, some common features corroborate findings observed in human cancer. There are several important similarities such as spontaneous tumor development and the influence of environmental factors that entail for more thoroughly designed comparative studies of human and dog cancer. Databases such as CCOGC (Canine and Comparative Oncology and Genomics Consortium) and BarkBase provide promising first steps and tools to elucidate the mechanisms behind canine cancer and support comparative studies between dogs and humans. However, these are only first steps and more research is necessary in order to better understand dogs as models to study epigenetics in cancer and drug development. We hope that the advancement of knowledge and technologies of epigenetic tools will aid the development of new targets and the advancement of drugs in veterinary oncology.

Table 1. Comparative studies regarding epigenetic changes in different human and canine cancer.

Human/Dog Comparative Epigenetic Studies			
Associated-Epigenetic Modification	**Findings**	**Type of tumor**	**Reference**
DNA Methylation	Genomic hypomethylation has been observed in Human and canine lymphoma and leukemia	Lymphoma; Leukemia	(PELHAM; IRWIN; KAY, 2003) x (YANG; WONG; NG, 2019)
DNA Methylation	Hypermethylation of Tumor suppressor gene *DLC1*	Non-Hodgkin's Lymphoma	(SHI et al., 2007) x (BRYAN et al., 2008)
DNA Methylation	Treatment with 5-AzaC reduces tumorigenicity in mammary cancer cells of Human, Dogs and cats.	Mammary Cancer	(HARMAN et al., 2016)
DNA Methylation	Methylation levels of *LINE-1* in circulating cell-free DNA (cfDNA) might be a useful diagnostic marker in human and canine mammary cancer.	Mammary Cancer	(LEE et al., 2019)
DNA Methylation	DNA methylation of microRNA-203 CpG islands contributes to Human and Canine Melanoma	Melanoma	(NOGUCHI et al., 2015b)
DNA Methylation	Hypermethylation of *TNF-α* promoter region was identified in human and canine melanoma cancer cells. Furthermore, the study observed a novel apoptosis-inducing mechanism of 5-aza-2-deoxycitidine.	Melanoma	(NOGUCHI et al., 2015a)

DNA Demethylation	*TET2* is commonly mutated in human hematopoietic tumors. However, the *TET2* mutation frequency in canine hematopoietic tumors, such as mast cell tumor and lymphomas, is very low.	Hematopoietic tumors	(RASMUSSEN et al., 2016)x(ZORZAN et al., 2015)x(MCDONALD et al., 2018)
DNA Methylation and histone modification	Combination of DNA methylation inhibitors and Chromatin-modified drugs is promising in Human and canine Osteosarcoma.	Osteosarcoma	(THAYANITHY et al., 2012)
Histone modifications	The HDAC inhibitor valproic acid can be used in combination with doxorubicin to treat human and canine osteosarcoma	Osteosarcoma	(WITTENBURG et al., 2011)
Histone modifications	HDAC inhibitor AR-42 induce apoptosis both in human and canine osteosarcoma cells.	Osteosarcoma	(MURAHARI et al., 2017)
MiRNAs	The role of miRNAs in human and mammary cancer.	Mammary cancer	(YU; CHEAH, 2017)
MiRNAs	MicroRNAs as tumor suppressors in human and canine melanoma cells	Melanoma	(NOGUCHI et al., 2013)
MiRNAs	Antioncogenic *miRNA-145* was downregulated in both human and canine melanoma cells	Melanoma	(NOGUCHI et al., 2012b)
LncRNAs	Oncogenic lncRNAs in human cancer, including *HOTAIR*, *MALAT1*, *PCA3*, *CASC15*, and *CASC20* are also annotated in dogs.	Different types of tissues	(WUCHER et al., 2017)x(LE BÉGUEC et al., 2018)x(HOEPPNER et al., 2014)
LncRNAs	A cross-species analysis of lncRNAs demonstrated that lncRNA associated with human diffuse large B-cell lymphoma (DLBLC) is also expressed in canine lymphoma	Lymphoma	(VERMA et al., 2015)

LncRNAs	LncRNA *ZEB2-AS*, *SOX21-AS1*, and *CASC15*, well-described in human cancer, was highly dysregulated in canine oral melanomas.	Melanoma	(BELTRAN et al., 2008)x(YANG et al., 2016)x(LESSARD et al., 2015)x(HITTE et al., 2019)

2.9 References

ADAMS, V. J. et al. Methods and mortality results of a health survey of purebred dogs in the UK. **Journal of Small Animal Practice**, v. 51, n. 10, p. 512–524, 2010.

ALBERTI, C.; COCHELLA, L. A framework for understanding the roles of miRNAs in animal development. **Development (Cambridge)**, v. 144, n. 14, p. 2548–2559, 2017.

ALBONICO, F. et al. The expression ratio of miR-17-5p and miR-155 correlates with grading in canine splenic lymphoma. **Veterinary Immunology and Immunopathology**, v. 155, n. 1–2, p. 117–123, 2013. Disponível em: <http://dx.doi.org/10.1016/j.vetimm.2013.06.018>.

ALVAREZ, H. et al. Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. **PLoS Genetics**, v. 7, n. 3, 2011.

ASADA, H. et al. Comprehensive analysis of miRNA and protein profiles within exosomes derived from canine lymphoid tumour cell lines. **PLoS ONE**, v. 14, n. 4, p. 1–15, 2019.

BAIONI, E. et al. Estimating canine cancer incidence: Findings from a population-based tumour registry in northwestern Italy. **BMC Veterinary Research**, v. 13, n. 1, p. 1–9, 2017.

BANNISTER, A. J.; KOUZARIDES, T. Regulation of chromatin by histone modifications. **Cell Research**, v. 21, n. 3, p. 381–395, 2011.

BAYLIN, S. B.; JONES, P. A. A decade of exploring the cancer epigenome-biological and translational implications. **Nature Reviews Cancer**, v. 11, n. 10, p. 726–734, 2011.

BELTRAN, M. et al. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. **Genes and Development**, v. 22, n. 6, p. 756–769, 2008.

BISWAS, S.; RAO, C. M. Epigenetic tools (The Writers, The Readers and The Erasers) and their implications in cancer therapy. **European Journal of Pharmacology**, v. 837, p. 8–24, 2018. Disponível em: <https://doi.org/10.1016/j.ejphar.2018.08.021>.

BOCKHORN, J. et al. Differentiation and loss of malignant character of spontaneous pulmonary metastases in patient-derived breast cancer models. **Cancer Research**, v. 74, n. 24, p. 7406–7417, 2014.

BOGGS, R. M. et al. MicroRNA expression in canine mammary cancer. **Mammalian Genome**, v. 19, n. 7–8, p. 561–569, 2008.

BØRRESEN, B. et al. Circulating let-7g is down-regulated in Bernese Mountain dogs with disseminated histiocytic sarcoma and carcinomas – a prospective study. **Veterinary and Comparative Oncology**, v. 15, n. 2, p. 525–533, 2017.

BRANDÃO, Y. de O. et al. DNA Methylation Status of the Estrogen Receptor α Gene in Canine Mammary Tumors. **Veterinary Pathology**, v. 55, n. 4, p. 510–516, 2018.

BRØNDEN, L. B. et al. Data from the Danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in Denmark. **Veterinary Record**, v. 166, n. 19, p. 586–590, 2010.

BRONZINI, I. et al. DNA methylation and targeted sequencing of methyltransferases

family genes in canine acute myeloid leukaemia, modelling human myeloid leukaemia. **Veterinary and Comparative Oncology**, v. 15, n. 3, p. 910–918, 2017.
BROWN, P. J.; MÜLLER, S. Open access chemical probes for epigenetic targets. **Future Medicinal Chemistry**, v. 7, n. 14, p. 1901–1917, 2015.
BROWN, R. et al. Poised epigenetic states and acquired drug resistance in cancer. **Nature Reviews Cancer**, v. 14, n. 11, p. 747–753, 2014.
BRYAN, J. N. et al. DNA methylation in cancer: techniques and preliminary evidence of hypermethylation in canine lymphoma. **Cancer Therapy**, v. 6, n. Cll, p. 137–148, 2008.
BRYAN, J. N. et al. Hypermethylation of the DLC1 CpG island does not alter gene expression in canine lymphoma. **BMC Genetics**, v. 10, p. 1–12, 2009.
BUKOWSKI, J. A.; WARTENBERG, D.; GOLDSCHMIDT, M. Environmental causes for sinonasal cancers in pet dogs, and their usefulness as sentinels of indoor cancer risk. **Journal of Toxicology and Environmental Health - Part A**, v. 54, n. 7, p. 579–591, 1998.
BULKOWSKA, M. et al. MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. **BMC Cancer**, v. 17, n. 1, p. 1–17, 2017.
CALIN, G. A. et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 24, p. 15524–15529, 2002.
CASCIELLO, F. et al. Functional role of G9a histone methyltransferase in cancer. **Frontiers in Immunology**, v. 6, n. SEP, p. 3–9, 2015.
CASCIONE, L. et al. Long non-coding RNAs as molecular signatures for canine B-cell lymphoma characterization. **Non-coding RNA**, v. 5, n. 3, 2019.
CEIFJAOEKCA, D. et al. Synthesis of a 2-deoxy-d-ribofuranosyl-s-azacytosine. v. 7, n. Ill, p. 2576–2578, 1964.
CHABOT, G. G. I lasma and Cerebrospinal Fluid Pharmacokinetics of 5-Aza-2 ' - d e o x y c y t i d i n e in Rabbits and D o g s 1. v. 43, n. February, p. 592–597, 1983.
CHAIYAWAT, P. et al. Expression patterns of class i histone deacetylases in osteosarcoma: A novel prognostic marker with potential therapeutic implications. **Modern Pathology**, v. 31, n. 2, p. 264–274, 2018. Disponível em: <http://dx.doi.org/10.1038/modpathol.2017.125>.
CHARLES, M. R. C. et al. Insights for the design of protein lysine methyltransferase G9a inhibitors. **Future Medicinal Chemistry**, v. 11, n. 9, p. 993–1014, 2019.
CHEUNG, H.-H. et al. DNA methylation of cancer genome. **Birth Defects Research Part C: Embryo Today: Reviews**, v. 87, n. 4, p. 335–350, dez. 2009. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf>.
CHOI, H. J. et al. Significance of EZH2 expression in canine mammary tumors. **BMC Veterinary Research**, v. 12, n. 1, p. 1–7, 2016. Disponível em: <http://dx.doi.org/10.1186/s12917-016-0789-2>.
CHOI, H. J. et al. EZH2 expression in naturally occurring canine tumors. **Comparative Medicine**, v. 68, n. 2, p. 148–155, 2018.
CHOI, J. W.; YOON, H. Y.; JEONG, S. W. Clinical outcomes of surgically managed spontaneous tumors in 114 client-owned dogs. **Immune Network**, v. 16, n. 2, p. 116–125, 2016.
CHUN, R. Lymphoma: Which Chemotherapy Protocol and Why? **Topics in Companion Animal Medicine**, v. 24, n. 3, p. 157–162, 2009. Disponível em: <http://dx.doi.org/10.1053/j.tcam.2009.03.003>.

COZZI, B. et al. Aging and veterinary care of cats, dogs, and horses through the records of three university veterinary hospitals. **Frontiers in Veterinary Science**, v. 4, n. FEB, p. 1–11, 2017.
CRAIG, K. K. L. et al. MicroRNA profiling in canine multicentric lymphoma. **PLoS ONE**, v. 14, n. 12, p. 1–24, 2019.
CRUZ CARDONA, J. A. et al. BCR-ABL translocation in a dog with chronic monocytic leukemia. **Veterinary Clinical Pathology**, v. 40, n. 1, p. 40–47, 2011.
CUI, M. et al. Circulating MicroRNAs in Cancer: Potential and Challenge. **Frontiers in Genetics**, v. 10, n. July, 2019.
DAMASKOS, C. et al. Histone deacetylase inhibitors: An attractive therapeutic strategy against breast cancer. **Anticancer Research**, v. 37, n. 1, p. 35–46, 2017.
DAWSON, M. A. The cancer epigenome: Concepts, challenges, and therapeutic opportunities. **Science**, v. 355, n. 6330, p. 1147–1152, 17 mar. 2017. Disponível em: <http://www.sciencemag.org/lookup/doi/10.1126/science.aam7304>.
DAWSON, M. A.; KOUZARIDES, T. Cancer epigenetics: From mechanism to therapy. **Cell**, v. 150, n. 1, p. 12–27, 2012. Disponível em: <http://dx.doi.org/10.1016/j.cell.2012.06.013>.
DIAS, J. N. R. et al. The histone deacetylase inhibitor panobinostat is a potent antitumor agent in canine diffuse large B-cell lymphoma. **Oncotarget**, v. 9, n. 47, p. 28586–28598, 2018a.
DIAS, J. N. R. et al. Establishment of a bioluminescent canine B-cell lymphoma xenograft model for monitoring tumor progression and treatment response in preclinical studies. **PLoS ONE**, v. 13, n. 12, p. 1–14, 2018b.
DIESCH, J. et al. A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers. **Clinical Epigenetics**, v. 8, n. 1, p. 1–11, 2016. Disponível em: <http://dx.doi.org/10.1186/s13148-016-0237-y>.
DOROSHOW, D. B.; EDER, J. P.; LORUSSO, P. M. BET inhibitors: A novel epigenetic approach. **Annals of Oncology**, v. 28, n. 8, p. 1776–1787, 2017. Disponível em: <http://dx.doi.org/10.1093/annonc/mdx157>.
DU, Z. et al. Integrative analyses reveal a long noncoding RNA-mediated sponge regulatory network in prostate cancer. **Nature Communications**, v. 7, 2016.
DUNHAM, I. et al. An integrated encyclopedia of DNA elements in the human genome. **Nature**, v. 489, n. 7414, p. 57–74, 2012.
EGENVALL, A. et al. Mortality in over 350,000 insured Swedish dogs from 1995-2000: II. Breed-specific age and survival patterns and relative risk for causes of death. **Acta Veterinaria Scandinavica**, v. 46, n. 3, p. 121–136, 2005.
EHRLICH, M. DNA methylation in cancer: Too much, but also too little. **Oncogene**, v. 21, n. 35 REV. ISS. 3, p. 5400–5413, 2002.
EHRLICH, M. DNA hypomethylation in cancer cells. **Epigenomics**, v. 1, n. 2, p. 239–259, dez. 2009. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf>.
ELSHAFAE, S. M. et al. The Effect of a Histone Deacetylase Inhibitor (AR-42) on Canine Prostate Cancer Growth and Metastasis. **Prostate**, v. 77, n. 7, p. 776–793, 2017.
EPIPHANIO, T. M. F. et al. Global DNA methylation of peripheral blood leukocytes from dogs bearing multicentric non-Hodgkin lymphomas and healthy dogs: A comparative study. **PLoS ONE**, v. 14, n. 3, p. 1–22, 2019.
ETO, S. et al. Anti-tumor effects of the histone deacetylase inhibitor vorinostat on canine urothelial carcinoma cells. **PLoS ONE**, v. 14, n. 6, p. 1–15, 2019.
FAN, H. et al. Low-dose decitabine-based chemoimmunotherapy for patients with refractory advanced solid tumors: A phase I/II report. **Journal of Immunology**

Research, v. 2014, 2014.
FANG, Y.; LIAO, G.; YU, B. LSD1/KDM1A inhibitors in clinical trials: Advances and prospects. **Journal of Hematology and Oncology**, v. 12, n. 1, p. 1–14, 2019.
FENAUX, P. et al. Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. **Journal of Clinical Oncology**, v. 28, n. 4, p. 562–569, 2010.
FENGER, J. M. et al. Overexpression of miR-9 in mast cells is associated with invasive behavior and spontaneous metastasis. **BMC Cancer**, v. 14, n. 1, p. 1–16, 2014.
FENGER, J. M. et al. MiR-9 is overexpressed in spontaneous canine osteosarcoma and promotes a metastatic phenotype including invasion and migration in osteoblasts and osteosarcoma cell lines. **BMC Cancer**, v. 16, n. 1, p. 1–19, 2016. Disponível em: <http://dx.doi.org/10.1186/s12885-016-2837-5>.
FENGER, J. M.; LONDON, C. A.; KISSEBERTH, W. C. Canine osteosarcoma: A naturally occurring disease to inform pediatric oncology. **ILAR Journal**, v. 55, n. 1, p. 69–85, 2014.
FERRARESSO, S. et al. Epigenetic silencing of TFPI-2 in canine diffuse large B-cell lymphoma. **PLoS ONE**, v. 9, n. 4, p. 1–10, 2014.
FERRARESSO, S. et al. DNA methylation profiling reveals common signatures of tumorigenesis and defines epigenetic prognostic subtypes of canine Diffuse Large B-cell Lymphoma. **Scientific Reports**, v. 7, n. 1, p. 1–11, 2017.
FIGHERA, R. A. et al. Causas de morte e razões para eutanásia de cães da Mesorregião do Centro Ocidental Rio-Grandense (1965-2004). **Pesquisa Veterinária Brasileira**, v. 28, n. 4, p. 223–230, 2008.
FIGUEIREDO, J. F. et al. Acute myeloblastic leukemia with associated BCR-ABL translocation in a dog. **Veterinary Clinical Pathology**, v. 41, n. 3, p. 362–368, set. 2012. Disponível em: <http://doi.wiley.com/10.1111/j.1939-165X.2012.00450.x>.
FISH, E. J. et al. Malignant canine mammary epithelial cells shed exosomes containing differentially expressed microRNA that regulate oncogenic networks. **BMC Cancer**, v. 18, n. 1, p. 1–20, 2018.
FLAVAHAN, W. A.; GASKELL, E.; BERNSTEIN, B. E. Epigenetic plasticity and the hallmarks of cancer. **Science**, v. 357, n. 6348, 2017.
FLEMING, J. M.; CREEVY, K. E.; PROMISLOW, D. E. L. Mortality in North American Dogs from 1984 to 2004: An Investigation into Age-, Size-, and Breed-Related Causes of Death. **Journal of Veterinary Internal Medicine**, v. 25, n. 2, p. 187–198, mar. 2011. Disponível em: <http://doi.wiley.com/10.1111/j.1939-1676.2011.0695.x>.
FLESNER, B. K.; KUMAR, S. R.; BRYAN, J. N. 6-Thioguanine and zebularine down-regulate DNMT1 and globally demethylate canine malignant lymphoid cells. **BMC Veterinary Research**, v. 10, n. 1, p. 1–7, 2014.
FORD, D. et al. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. **American Journal of Human Genetics**, v. 62, n. 3, p. 676–689, 1998.
FOSSEY, S. L. et al. Characterization of STAT3 activation and expression in canine and human osteosarcoma. **BMC Cancer**, v. 9, p. 1–15, 2009.
FRAGA, M. F. et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. **Nature Genetics**, v. 37, n. 4, p. 391–400, 2005.
FUJIWARA-IGARASHI, A. et al. Inhibition of p16 tumor suppressor gene expression via promoter hypermethylation in canine lymphoid tumor cells. **Research in Veterinary Science**, v. 97, n. 1, p. 60–63, 2014. Disponível em:

<http://dx.doi.org/10.1016/j.rvsc.2014.04.008>.
FUJIWARA-IGARASHI, A. et al. Expression profile of circulating serum microRNAs in dogs with lymphoma. **Veterinary Journal**, v. 205, n. 2, p. 317–321, 2015. Disponível em: <http://dx.doi.org/10.1016/j.tvjl.2015.04.029>.
FULKERSON, C. M. et al. Pharmacokinetics and toxicity of the novel oral demethylating agent zebularine in laboratory and tumor bearing dogs. **Veterinary and Comparative Oncology**, v. 15, n. 1, p. 226–236, 2017.
GANESAN, A. et al. The timeline of epigenetic drug discovery: from reality to dreams. **Clinical epigenetics**, v. 11, n. 1, p. 174, 2019.
GARCÍA, E. et al. Epidemiology of tumors in dogs in the capital of the state of Mexico from 2002-2016. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, v. 71, n. 4, p. 1085–1092, 2019.
GARDNER, H. L. et al. Canine osteosarcoma genome sequencing identifies recurrent mutations in DMD and the histone methyltransferase gene SETD2. **Communications Biology**, v. 2, n. 1, p. 1–13, 2019. Disponível em: <http://dx.doi.org/10.1038/s42003-019-0487-2>.
GARDNER, H. L.; FENGER, J. M.; LONDON, C. A. Dogs as a Model for Cancer. **Annual Review of Animal Biosciences**, v. 4, n. 1, p. 199–222, 15 fev. 2016. Disponível em: <http://www.annualreviews.org/doi/10.1146/annurev-animal-022114-110911>.
GAVAZZA, A. et al. Association between canine malignant lymphoma, living in industrial areas, and use of chemicals by dog owners. **Journal of Veterinary Internal Medicine**, v. 15, n. 3, p. 190–195, 2001.
GLOZAK, M. A.; SETO, E. Histone deacetylases and cancer. **Oncogene**, v. 26, n. 37, p. 5420–5432, 2007.
GOLDSCHMIDT, M. H. et al. Classification and grading of canine mammary tumors. **Veterinary Pathology**, v. 48, n. 1, p. 117–131, 2011.
GRANT, C. et al. Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors. **Expert Review of Anticancer Therapy**, v. 10, n. 7, p. 997–1008, 10 jul. 2010. Disponível em: <http://www.tandfonline.com/doi/full/10.1586/era.10.88>.
GREENBERG, M. V. C.; BOURC'HIS, D. The diverse roles of DNA methylation in mammalian development and disease. **Nature Reviews Molecular Cell Biology**, v. 20, n. 10, p. 590–607, 2019. Disponível em: <http://dx.doi.org/10.1038/s41580-019-0159-6>.
GRIMES, J. A. et al. A comparison of microRNA expression profiles from splenic hemangiosarcoma, splenic nodular hyperplasia, and normal spleens of dogs. **BMC Veterinary Research**, v. 12, n. 1, p. 1–12, 2016. Disponível em: <http://dx.doi.org/10.1186/s12917-016-0903-5>.
GRÜNTZIG, K. et al. The Swiss canine cancer registry: A retrospective study on the occurrence of tumours in dogs in Switzerland from 1955 to 2008. **Journal of Comparative Pathology**, v. 152, n. 2–3, p. 161–171, 2015.
GULATI, N.; BÉGUELIN, W.; GIULINO-ROTH, L. Enhancer of zeste homolog 2 (EZH2) inhibitors. **Leukemia and Lymphoma**, v. 59, n. 7, p. 1574–1585, 2018.
GUPTA, K. Epidemiological Studies on Canine Mammary Tumour and its Relevance for Breast Cancer Studies. **IOSR Journal of Pharmacy (IOSRPHR)**, v. 2, n. 2, p. 322–333, 2012.
GUPTA, K.; SOOD, N.; UPPAL, S. Epidemiological studies on canine mammary tumour and its relevance for breast cancer studies. **IOSR Journal of Pharmacy**, v. 2, n. 2, p. 322–333, 2012. Disponível em:

<http://www.iosrphr.org/papers/v2i2/ZJ022322333.pdf>.
GUPTA, R. A. et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. **Nature**, v. 464, n. 7291, p. 1071–1076, 2010.
GUTSCHNER, T. et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. **Cancer Research**, v. 73, n. 3, p. 1180–1189, 2013.
HAHN, N. M. et al. Subcutaneous 5-Azacitidine Treatment of Naturally Occurring Canine Urothelial Carcinoma: A Novel Epigenetic Approach to Human Urothelial Carcinoma Drug Development. **Journal of Urology**, v. 187, n. 1, p. 302–309, jan. 2012. Disponível em: <http://www.jurology.com/doi/10.1016/j.juro.2011.09.010>.
HALKIDOU, K. et al. Upregulation and Nuclear Recruitment of HDACl in Hormone Refractory Prostate Cancer. **Prostate**, v. 59, n. 2, p. 177–189, 2004.
HAN, L. et al. DNA methylation regulates microRNA expression. **Cancer Biology and Therapy**, v. 6, n. 8, p. 1284–1288, 2007.
HAO, Y. et al. Prediction of long noncoding RNA functions with co-expression network in esophageal squamous cell carcinoma. **BMC Cancer**, v. 15, n. 1, p. 1–10, 2015.
HARMAN, R. M. et al. A Comparative Study on the In Vitro Effects of the DNA Methyltransferase Inhibitor 5-Azacytidine (5-AzaC) in Breast/Mammary Cancer of Different Mammalian Species. **Journal of Mammary Gland Biology and Neoplasia**, v. 21, n. 1–2, p. 51–66, 2016. Disponível em: <http://dx.doi.org/10.1007/s10911-016-9350-y>.
HAYES, H. M.; HOOVER, R.; TARONE, R. E. Bladder cancer in pet dogs: A sentinel for environmental cancer? **American Journal of Epidemiology**, v. 114, n. 2, p. 229–233, 1981.
HEISHIMA, K. et al. Circulating microRNA-214 and -126 as potential biomarkers for canine neoplastic disease. **Scientific Reports**, v. 7, n. 1, p. 1–14, 2017.
HEISHIMA, K. et al. Prognostic significance of circulating microRNA-214 and -126 in dogs with appendicular osteosarcoma receiving amputation and chemotherapy. **BMC Veterinary Research**, v. 15, n. 1, p. 1–13, 2019.
HENDRIX, M. J. C. et al. Reprogramming metastatic tumour cells with embryonic microenvironments. **Nature Reviews Cancer**, v. 7, n. 4, p. 246–255, 2007.
HERRERA, C. L. et al. Peroxisome proliferator activated receptor γ protein expression is asymmetrically distributed in primary lung tumor and metastatic to lung osteosarcoma samples and does not correlate with gene methylation. **BMC Veterinary Research**, v. 11, n. 1, p. 1–11, 2015. Disponível em: <http://dx.doi.org/10.1186/s12917-015-0547-x>.
HITTE, C. et al. Genome-wide analysis of long non-coding RNA profiles in canine oral melanomas. **Genes**, v. 10, n. 6, p. 1–14, 2019.
HOEPPNER, M. P. et al. An improved canine genome and a comprehensive catalogue of coding genes and non-coding transcripts. **PLoS ONE**, v. 9, n. 3, 2014.
HOSONO, Y. et al. Oncogenic Role of THOR, a Conserved Cancer/Testis Long Non-coding RNA. **Cell**, v. 171, n. 7, p. 1559- 1572.e20, dez. 2017. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0092867417314307>.
HSU, W. L. et al. Increased survival in dogs with malignant mammary tumours overexpressing HER-2 protein and detection of a silent single nucleotide polymorphism in the canine HER-2 gene. **Veterinary Journal**, v. 180, n. 1, p. 116–123, 2009. Disponível em: <http://dx.doi.org/10.1016/j.tvjl.2007.10.013>.
HUSTON, A. et al. Probing the epigenome. **Nature Chemical Biology**, v. 11, n. 8, p. 542–545, 2015.

ILIOU, M. S. et al. Impaired DICER1 function promotes stemness and metastasis in colon cancer. **Oncogene**, v. 33, n. 30, p. 4003–4015, 2014.
IM, K. S. et al. Breed-related differences in altered BRCA1 expression, phenotype and subtype in malignant canine mammary tumors. **Veterinary Journal**, v. 195, n. 3, p. 366–372, 2013. Disponível em: <http://dx.doi.org/10.1016/j.tvjl.2012.07.014>.
INOUE, M. et al. A current life table and causes of death for insured dogs in Japan. **Preventive Veterinary Medicine**, v. 120, n. 2, p. 210–218, 2015. Disponível em: <http://dx.doi.org/10.1016/j.prevetmed.2015.03.018>.
IQBAL, N.; IQBAL, N. Human Epidermal Growth Factor Receptor 2 (HER2) in Cancers: Overexpression and Therapeutic Implications. **Molecular Biology International**, v. 2014, p. 1–9, 2014.
IYER, N. G.; ÖZDAG, H.; CALDAS, C. p300/CBP and cancer. **Oncogene**, v. 23, n. 24, p. 4225–4231, 2004.
JACKSON, K. et al. DNA hypomethylation is prevalent even in low-grade breast cancers. **Cancer Biology and Therapy**, v. 3, n. 12, p. 1225–1231, 2004.
JONES, P. A. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. **Nature Reviews Genetics**, v. 13, n. 7, p. 484–492, 2012.
KAKE, S. et al. The role of SET/I2PP2A in canine mammary tumors. **Scientific Reports**, v. 7, n. 1, p. 1–11, 2017.
KAMINSKAS, E. et al. FDA Drug Approval Summary: Azacitidine (5-azacytidine, Vidaza ™) for Injectable Suspension . **The Oncologist**, v. 10, n. 3, p. 176–182, 2005.
KANTARJIAN, H. M. et al. Guadecitabine (SGI-110) in treatment-naive patients with acute myeloid leukaemia: phase 2 results from a multicentre, randomised, phase 1/2 trial. **The Lancet Oncology**, v. 18, n. 10, p. 1317–1326, out. 2017. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S1470204517305764>.
KAUSHIK, D. et al. Histone deacetylase inhibitors in castration-resistant prostate cancer: Molecular mechanism of action and recent clinical trials. **Therapeutic Advances in Urology**, v. 7, n. 6, p. 388–395, 2015.
KEBEDE, A. F.; SCHNEIDER, R.; DAUJAT, S. Novel types and sites of histone modifications emerge as players in the transcriptional regulation contest. **FEBS Journal**, v. 282, n. 9, p. 1658–1674, 2015.
KELSEY, J. L.; MOORE, A. S.; GLICKMAN, L. T. Epidemiologic studies of risk factors for cancer in pet dogs. **Epidemiologic Reviews**, v. 20, n. 2, p. 204–217, 1998.
KENT, M. S. et al. MicroRNA profiling of dogs with transitional cell carcinoma of the bladder using blood and urine samples. **BMC Veterinary Research**, v. 13, n. 1, p. 1–13, 2017.
KHANNA, C. et al. The dog as a cancer model. **Nature Biotechnology**, v. 24, n. 9, p. 1065–1066, set. 2006. Disponível em: <http://www.nature.com/articles/nbt0906-1065b>.
KIM, J. H. et al. Downregulation of gelsolin and retinoic acid receptor β expression in gastric cancer tissues through histone deacetylase 1. **Journal of Gastroenterology and Hepatology (Australia)**, v. 19, n. 2, p. 218–224, 2004.
KIM, K. H.; ROBERTS, C. W. M. Targeting EZH2 in cancer. **Nature Medicine**, v. 22, n. 2, p. 128–134, 4 fev. 2016. Disponível em: <http://www.nature.com/articles/nm.4036>.
KIRPENSTEIJN, J. et al. TP53 gene mutations in canine osteosarcoma. **Veterinary Surgery**, v. 37, n. 5, p. 454–460, 2008.
KLOOSTERMAN, W. P.; PLASTERK, R. H. A. The Diverse Functions of MicroRNAs in Animal Development and Disease. **Developmental Cell**, v. 11, n. 4, p. 441–450, 2006.

KOBAYASHI, M. et al. Microrna expression profiling in canine prostate cancer. **Journal of Veterinary Medical Science**, v. 79, n. 4, p. 719–725, 2017.
KOUZARIDES, T. Chromatin Modifications and Their Function. **Cell**, v. 128, n. 4, p. 693–705, 2007.
KUMAGAI, T. et al. Histone deacetylase inhibitor, suberoylanilide hydroxamic acid (Vorinostat, SAHA) profoundly inhibits the growth of human pancreatic cancer cells. **International Journal of Cancer**, v. 121, n. 3, p. 656–665, 2007.
LAWRENCE, M.; DAUJAT, S.; SCHNEIDER, R. Lateral Thinking: How Histone Modifications Regulate Gene Expression. **Trends in Genetics**, v. 32, n. 1, p. 42–56, 2016. Disponível em: <http://dx.doi.org/10.1016/j.tig.2015.10.007>.
LE BÉGUEC, C. et al. Characterisation and functional predictions of canine long non-coding RNAs. **Scientific Reports**, v. 8, n. 1, p. 1–12, 2018.
LEDET, M. M. et al. BB-Cl-Amidine as a novel therapeutic for canine and feline mammary cancer via activation of the endoplasmic reticulum stress pathway. **BMC Cancer**, v. 18, n. 1, p. 1–13, 2018.
LEE, H. Z. et al. FDA approval: Belinostat for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma. **Clinical Cancer Research**, v. 21, n. 12, p. 2666–2670, 2015.
LEE, K. H. et al. Methylation of LINE-1 in cell-free DNA serves as a liquid biopsy biomarker for human breast cancers and dog mammary tumors. **Scientific Reports**, v. 9, n. 1, p. 1–10, 2019.
LESSARD, L. et al. The CASC15 Long Intergenic Noncoding RNA Locus Is Involved in Melanoma Progression and Phenotype Switching. **Journal of Investigative Dermatology**, v. 135, n. 10, p. 2464–2474, out. 2015. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0022202X15418111>.
LEUCCI, E. et al. Melanoma addiction to the long non-coding RNA SAMMSON. **Nature**, v. 531, n. 7595, p. 518–522, 23 mar. 2016. Disponível em: <http://www.nature.com/articles/nature17161>.
LI, A. et al. Correction: HDAC5, a potential therapeutic target and prognostic biomarker, promotes proliferation, invasion and migration in human breast cancer [Oncotarget., 7, 25 (2016) (37966-37978)] DOI:10.18632/oncotarget.9274. **Oncotarget**, v. 8, n. 18, p. 30619–30620, 2017.
LI, Y. et al. HBXIP and LSD1 scaffolded by lncRNA hotair mediate transcriptional activation by c-Myc. **Cancer Research**, v. 76, n. 2, p. 293–304, 2016.
LIN, T. Y. et al. AR-42, a novel HDAC inhibitor, exhibits biologic activity against malignant mast cell lines via down-regulation of constitutively activated Kit. **Blood**, v. 115, n. 21, p. 4217–4225, 2010.
LIU, D. et al. Molecular homology and difference between spontaneous canine mammary cancer and human breast cancer. **Cancer Research**, v. 74, n. 18, p. 5045–5056, 2014.
LONSDALE, J. et al. The Genotype-Tissue Expression (GTEx) project. **Nature Genetics**, v. 45, n. 6, p. 580–585, 2013.
LOPEZ, C. M. et al. MiR-34a regulates the invasive capacity of canine osteosarcoma cell lines. **PLoS ONE**, v. 13, n. 1, p. 1–23, 2018.
LUGER, K. et al. Crystal structure of the nucleosome core particle at 2.8 Å resolution. **Nature**, v. 389, n. 6648, p. 251–260, 1997.
LUU, T. et al. Phase IB trial of ixabepilone and vorinostat in metastatic breast cancer. **Breast Cancer Research and Treatment**, v. 167, n. 2, p. 469–478, 2018.
MA, X. et al. Targeting CD146 in combination with vorinostat for the treatment of ovarian cancer cells. **Oncology Letters**, v. 13, n. 3, p. 1681–1687, 2017.

MACIOTTA, S.; MEREGALLI, M.; TORRENTE, Y. The involvement of microRNAs in neurodegenerative diseases. **Frontiers in Cellular Neuroscience**, v. 7, n. DEC, p. 1–17, 2013.
MANN, B. S. et al. FDA Approval Summary: Vorinostat for Treatment of Advanced Primary Cutaneous T-Cell Lymphoma. **The Oncologist**, v. 12, n. 10, p. 1247–1252, 2007.
MCDONALD, J. T. et al. Correction: Comparative oncology DNA sequencing of canine T cell lymphoma via human hotspot panel [Oncotarget., 9, (2018) (22693-22702)] doi: 10.18632/oncotarget.25209. **Oncotarget**, v. 9, n. 70, p. 33441, 2018.
MEGQUIER, K. et al. Barkbase: Epigenomic annotation of canine genomes. **Genes**, v. 10, n. 6, 2019.
MERLO, D. F. et al. Cancer Incidence in Pet Dogs: Findings of the Animal Tumor Registry of Genoa, Italy. **Journal of Veterinary Internal Medicine**, v. 22, n. 4, p. 976–984, jul. 2008. Disponível em: <http://doi.wiley.com/10.1111/j.1939-1676.2008.0133.x>.
MICHALAK, E. M.; VISVADER, J. E. Dysregulation of histone methyltransferases in breast cancer – Opportunities for new targeted therapies? **Molecular Oncology**, v. 10, n. 10, p. 1497, 2016.
MISKA, E. A. How microRNAs control cell division, differentiation and death. **Current Opinion in Genetics and Development**, v. 15, n. 5 SPEC. ISS., p. 563–568, 2005.
MITHRAPRABHU, S. et al. Dysregulated Class I histone deacetylases are indicators of poor prognosis in multiple myeloma. **Epigenetics**, v. 9, n. 11, p. 1511–1520, 2014.
MONDAL, T. et al. MEG3 long noncoding RNA regulates the TGF-β pathway genes through formation of RNA-DNA triplex structures. **Nature Communications**, v. 6, 2015.
MORIMOTO, C. Y. et al. Evaluation of the global DNA methylation in canine mast cell tumour samples by immunostaining of 5-methyl cytosine. **Veterinary and Comparative Oncology**, v. 15, n. 3, p. 1014–1018, 2017.
MORTARINO, M. et al. Identification of suitable endogenous controls and differentially expressed microRNAs in canine fresh-frozen and FFPE lymphoma samples. **Leukemia Research**, v. 34, n. 8, p. 1070–1077, 2010. Disponível em: <http://dx.doi.org/10.1016/j.leukres.2009.10.023>.
MUDALIAR, M. A. V. et al. Comparative Gene Expression Profiling Identifies Common Molecular Signatures of NF-κB Activation in Canine and Human Diffuse Large B Cell Lymphoma (DLBCL). **PLoS ONE**, v. 8, n. 9, 2013.
MULJO, S. A.; ZHU, J.; ZHAO, K. Expression and regulation of lincRNAs during T cell development and differentiation. **Nature immunology**, v. 14, n. 11, p. 1190–1198, 2013.
MÜLLER, S.; BROWN, P. J. Epigenetic chemical probes. **Clinical Pharmacology and Therapeutics**, v. 92, n. 6, p. 689–693, 2012.
MURAHARI, S. et al. Sensitivity of osteosarcoma cells to HDAC inhibitor AR-42 mediated apoptosis. **BMC Cancer**, v. 17, n. 1, p. 1–11, 2017. Disponível em: <http://dx.doi.org/10.1186/s12885-017-3046-6>.
NAGAMINE, M. K. et al. In vitro inhibitory effect of trichostatin A on canine grade 3 mast cell tumor. **Veterinary Research Communications**, v. 35, n. 6, p. 391–399, 2011.
NARAYAN, A. et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. **International Journal of Cancer**, v. 77, n. 6, p. 833–838, 1998.
NERVI, C.; DE MARINIS, E.; CODACCI-PISANELLI, G. Epigenetic treatment of solid tumours: A review of clinical trials. **Clinical Epigenetics**, v. 7, n. 1, 2015.

Disponível em: <http://dx.doi.org/10.1186/s13148-015-0157-2>.
NGUYEN, C. T. et al. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. **Cancer Research**, v. 62, n. 22, p. 6456–6461, 2002.
NICHOL, J. N. et al. H3K27 Methylation. In: **Physiology & behavior**. [s.l: s.n.]176p. 59–95.
NOGUCHI, S. et al. Anti-oncogenic microRNA-203 induces senescence by targeting E2F3 protein in human melanoma cells. **Journal of Biological Chemistry**, v. 287, n. 15, p. 11769–11777, 2012a.
NOGUCHI, S. et al. Comparative study of anti-oncogenic MicroRNA-145 in canine and human malignant melanoma. **Journal of Veterinary Medical Science**, v. 74, n. 1, p. 1–8, 2012b.
NOGUCHI, S. et al. MicroRNAs as tumour suppressors in canine and human melanoma cells and as a prognostic factor in canine melanomas. **Veterinary and Comparative Oncology**, v. 11, n. 2, p. 113–123, 2013.
NOGUCHI, S. et al. A novel apoptosis-inducing mechanism of 5-aza-2'-deoxycitidine in melanoma cells: Demethylation of TNF-α and activation of FOXO1. **Cancer Letters**, v. 369, n. 2, p. 344–353, 2015a. Disponível em: <http://dx.doi.org/10.1016/j.canlet.2015.08.023>.
NOGUCHI, S. et al. DNA methylation contributes toward silencing of antioncogenic microRNA-203 in human and canine melanoma cells. **Melanoma Research**, v. 25, n. 5, p. 390–398, 2015b.
O'DONNELL, K. A. et al. c-Myc-regulated microRNAs modulate E2F1 expression. **Nature**, v. 435, n. 7043, p. 839–843, 2005.
OSSENKOPPELE, G.; LOWENBERG, B. How I treat the older patient with AML. **Blood**, v. 125, n. 5, p. 767–774, 2015. Disponível em: <http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=medl&NEWS=N&AN=25515963>.
OTTAVIANO, Y. L. et al. Methylation of the Estrogen Receptor Gene CpG Island Marks Loss of Estrogen Receptor Expression in Human Breast Cancer Cells. **Cancer Research**, v. 54, n. 10, p. 2552–2555, 1994.
PAOLONI, M.; KHANNA, C. Translation of new cancer treatments from pet dogs to humans. **Nature Reviews Cancer**, v. 8, n. 2, p. 147–156, 2008.
PASTOR, N. et al. Epidemiological study of canine mammary tumors: Age, breed, size and malignancy. **Austral Journal of Veterinary Sciences**, v. 50, n. 3, p. 143–147, 2018.
PELHAM, J. T.; IRWIN, P. J.; KAY, P. H. Genomic hypomethylation in neoplastic cells from dogs with malignant lymphoproliferative disorders. **Research in Veterinary Science**, v. 74, n. 1, p. 101–104, 2003.
PENG, Y.; CROCE, C. M. The role of microRNAs in human cancer. **Signal Transduction and Targeted Therapy**, v. 1, n. November 2015, 2016.
PLASSAIS, J. et al. A Point Mutation in a lincRNA Upstream of GDNF Is Associated to a Canine Insensitivity to Pain: A Spontaneous Model for Human Sensory Neuropathies. **PLoS Genetics**, v. 12, n. 12, p. 1–21, 2016.
POPHALI, P. A. et al. High level MYC amplification in B-cell lymphomas: is it a marker of aggressive disease? **Blood Cancer Journal**, v. 10, n. 1, 2020. Disponível em: <http://dx.doi.org/10.1038/s41408-019-0271-z>.
PRABHU, J. S. et al. The epigenetic silencing of the estrogen receptor (ER) by hypermethylation of the ESR1 promoter is seen predominantly in triple-negative breast cancers in Indian women. **Tumor Biology**, v. 33, n. 2, p. 315–323, 2012.

QIU, H. B. et al. Promoter mutation and reduced expression of BRCA1 in canine mammary tumors. **Research in Veterinary Science**, v. 103, p. 143–148, 2015. Disponível em: <http://dx.doi.org/10.1016/j.rvsc.2015.10.003>.
QIU, H.; LIN, D. Roles of DNA mutation in the coding region and DNA methylation in the 5′ flanking region of BRCA1 in canine mammary tumors. **Journal of Veterinary Medical Science**, v. 78, n. 6, p. 943–949, 2016.
QUEIROGA, F. L. et al. Canine mammary tumours as a model to study human breast cancer: most recent findings. **In vivo (Athens, Greece)**, v. 25, n. 3, p. 455–465, 2011.
QUINTÁS-CARDAMA, A.; CORTES, J. Molecular Biology of Bcr-Abl1-Positive Chronic Myeloid Leukemia. **Blood**, v. 113, n. 8, p. 1619–1630, 19 fev. 2009. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/18827185>.
RAEDLER, B. L. a; WRITER, M. Monoclonal Antibody Approved for Patients with Relapsed Multiple Myeloma. **American Health & Drug Benefits**, v. 9, n. March, p. 84–89, 2016.
RAHMAN, M. M. et al. Micro RNA transcriptome profile in canine oral melanoma. **International Journal of Molecular Sciences**, v. 20, n. 19, 2019.
RASMUSSEN, K. D.; HELIN, K. Role of TET enzymes in DNA methylation, development, and cancer. **Genes and Development**, v. 30, n. 7, p. 733–750, 2016.
REBHUN, R. B. et al. CHOP chemotherapy for the treatment of canine multicentric T-cell lymphoma. **Veterinary and Comparative Oncology**, v. 9, n. 1, p. 38–44, 2011.
RIVERA, P. J. et al. Mammary tumor development in dogs is associated with BRCA1 and BRCA2. **Cancer Research**, v. 69, n. 22, p. 8770–8774, 2009.
RIZZO, V. L.; LEVINE, C. B.; WAKSHLAG, J. J. The effects of sulforaphane on canine osteosarcoma proliferation and invasion. **Veterinary and Comparative Oncology**, v. 15, n. 3, p. 718–730, 2017.
ROADMAP EPIGENOMICS CONSORTIUM et al. Integrative analysis of 111 reference human epigenomes. **Nature**, v. 518, n. 7539, p. 317–329, 2015.
ROBEY, R. W. et al. Revisiting the role of ABC transporters in multidrug-resistant cancer. **Nature Reviews Cancer**, v. 18, n. 7, p. 452–464, 2018. Disponível em: <http://dx.doi.org/10.1038/s41568-018-0005-8>.
ROS, S. Da et al. Validation of epigenetic mechanisms regulating gene expression in canine B-cell lymphoma: An in vitro and in vivo approach. **PLoS ONE**, v. 13, n. 12, p. 1–19, 2018.
ROWBOTHAM, S. P. et al. H3K9 methyltransferases and demethylases control lung tumor-propagating cells and lung cancer progression. **Nature Communications**, v. 9, n. 1, 2018.
SABA, H. I. Decitabine in the treatment of myelodysplastic syndromes. **Therapeutics and Clinical Risk Management**, v. 3, n. 5, p. 807–817, 2007.
SAKTHIKUMAR, S. et al. SETD2 is recurrently mutated in whole-exome sequenced canine osteosarcoma. **Cancer Research**, v. 78, n. 13, p. 3421–3431, 2018.
SATO, M. et al. Hypermethylation of the death-associated protein kinase CpG island in canine B-cell lymphoid tumors. **Veterinary Immunology and Immunopathology**, v. 161, n. 3–4, p. 222–231, 2014. Disponível em: <http://dx.doi.org/10.1016/j.vetimm.2014.08.011>.
SATO, M. et al. Prognostic significance of hypermethylation of death-associated protein kinase (DAPK) gene CpG island in dogs with high-grade B-cell lymphoma. **Veterinary and Comparative Oncology**, v. 16, n. 3, p. 409–415, 2018.
SBOROV, D. W. et al. A phase 1 trial of the HDAC inhibitor AR-42 in patients with multiple myeloma and T- and B-cell lymphomas. **Leukemia & Lymphoma**, v. 58, n. 10, p. 2310–2318, 3 out. 2017. Disponível em:

<https://www.tandfonline.com/doi/full/10.1080/10428194.2017.1298751>.
SCHEER, S. et al. A chemical biology toolbox to study protein methyltransferases and epigenetic signaling. **Nature Communications**, v. 10, n. 1, p. 1–14, 2019. Disponível em: <http://dx.doi.org/10.1038/s41467-018-07905-4>.
SHI, H. et al. Discovery of novel epigenetic markers in non-Hodgkin's lymphoma. **Carcinogenesis**, v. 28, n. 1, p. 60–70, 2007.
SINGH, V.; CAPALASH, P. S. and N. **DNA Methyltransferase-1 Inhibitors as Epigenetic Therapy for CancerCurrent Cancer Drug Targets**, 2013. . Disponível em: <http://www.eurekaselect.com/node/109882/article>.
SLACK, F. J.; CHINNAIYAN, A. M. The Role of Non-coding RNAs in Oncology. **Cell**, v. 179, n. 5, p. 1033–1055, 2019. Disponível em: <https://doi.org/10.1016/j.cell.2019.10.017>.
SORENMO, K. Canine mammary gland tumors. **Veterinary Clinics of North America - Small Animal Practice**, v. 33, n. 3, p. 573–596, 2003.
STARKEY, M. P. et al. Metastasis-associated microRNA expression in canine uveal melanoma. **Veterinary and Comparative Oncology**, v. 16, n. 1, p. 81–89, 2018.
STEIN, E. M. et al. The DOT1L inhibitor pinometostat reduces H3K79 methylation and has modest clinical activity in adult acute leukemia. **Blood**, v. 131, n. 24, p. 2662–2669, 2018.
STRANDBERG, J. D.; GOODMAN, D. G. Animal model: Canine mammary neoplasia. **American Journal of Pathology**, v. 75, n. 1, p. 225–228, 1974.
TEFFERI, A. et al. Frequent TET2 mutations in systemic mastocytosis: Clinical, KITD816V and FIP1L1-PDGFRA correlates. **Leukemia**, v. 23, n. 5, p. 900–904, 2009.
THAYANITHY, V. et al. Combinatorial Treatment of DNA and Chromatin-Modifying Drugs Cause Cell Death in Human and Canine Osteosarcoma Cell Lines. **PLoS ONE**, v. 7, n. 9, 2012.
THOMAS, R. et al. Influence of genetic background on tumor karyotypes: Evidence for breed-associated cytogenetic aberrations in canine appendicular osteosarcoma. **Chromosome Research**, v. 17, n. 3, p. 365–377, 2009.
THOMAS, R. et al. Refining tumor-associated aneuploidy through 'genomic recoding' of recurrent DNA copy number aberrations in 150 canine non-Hodgkin lymphomas. **Leukemia & Lymphoma**, v. 52, n. 7, p. 1321–1335, 7 jul. 2011. Disponível em: <http://www.tandfonline.com/doi/full/10.3109/10428194.2011.559802>.
THOMSON, J. M. et al. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. **Genes and Development**, v. 20, n. 16, p. 2202–2207, 2006.
TKACZYNSKI, T.; ŠMEJKAL, J.; ŠORM, F. Nucleic acids components and their analogues. L. Synthesis of 1-β-D-xylofuranosyl-6-azauracil. **Collection of Czechoslovak Chemical Communications**, v. 29, n. 7, p. 1736–1738, 1964.
TOMIYASU, H. et al. Epigenetic regulation of the ABCB1 gene in drug-sensitive and drug-resistant lymphoid tumour cell lines obtained from canine patients. **Veterinary Journal**, v. 199, n. 1, p. 103–109, 2014a. Disponível em: <http://dx.doi.org/10.1016/j.tvjl.2013.10.022>.
TOMIYASU, H. et al. Evaluation of DNA methylation profiles of the CpG island of the ABCB1 gene in dogs with lymphoma. **American Journal of Veterinary Research**, v. 75, n. 9, p. 835–841, 2014b.
TSAI, M.-C. et al. Long Noncoding RNA as Modular Scaffold of Histone Modification Complexes. **Science**, v. 329, n. 5992, p. 689–693, 6 ago. 2010. Disponível em: <https://www.sciencemag.org/lookup/doi/10.1126/science.1192002>.
TSUJI, S. et al. Efficacy of an anti-cancer strategy targeting set in canine osteosarcoma. **Journal of Veterinary Medical Science**, v. 81, n. 10, p. 1424–1430, 2019.

TU, W. B. et al. MYC Interacts with the G9a Histone Methyltransferase to Drive Transcriptional Repression and Tumorigenesis. **Cancer Cell**, v. 34, n. 4, p. 579- 595.e8, 2018. Disponível em: <https://doi.org/10.1016/j.ccell.2018.09.001>.
USHIO, N. et al. Identification of dysregulated microRNAs in canine malignant melanoma. **Oncology Letters**, v. 17, n. 1, p. 1080–1088, 2019.
VAN LEEUWEN, I. S. et al. P53 gene mutations in osteosarcomas in the dog. **Cancer Letters**, v. 111, n. 1–2, p. 173–178, 1997.
VERMA, A. et al. Transcriptome sequencing reveals thousands of novel long non-coding RNAs in B cell lymphoma. **Genome Medicine**, v. 7, n. 1, p. 1–17, 2015. Disponível em: <http://dx.doi.org/10.1186/s13073-015-0230-7>.
VIGUSHIN, D. M. et al. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. **Clinical Cancer Research**, v. 7, n. 4, p. 971–976, 2001.
WAN, Y. C. E.; LIU, J.; CHAN, K. M. Histone H3 Mutations in Cancer. **Current Pharmacology Reports**, v. 4, n. 4, p. 292–300, 2018.
WANG, Z. et al. lncRNA Epigenetic Landscape Analysis Identifies EPIC1 as an Oncogenic lncRNA that Interacts with MYC and Promotes Cell-Cycle Progression in Cancer. **Cancer Cell**, v. 33, n. 4, p. 706- 720.e9, 2018.
WATANABE, M. et al. Tumor cell growth inhibition and cell differentiation analysis in a canine mammary tumor cell line (MCM-B2) treated with four chemical reagents. **Journal of Veterinary Medical Science**, v. 71, n. 11, p. 1413–1417, 2009.
WEICHERT, W. et al. Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis. **The Lancet Oncology**, v. 9, n. 2, p. 139–148, 2008a.
WEICHERT, W. et al. Expression of class I histone deacetylases indicates poor prognosis in endometrioid subtypes of ovarian and endometrial carcinomas. **Neoplasia**, v. 10, n. 9, p. 1021–1027, 2008b.
WEISWALD, L. B.; BELLET, D.; DANGLES-MARIE, V. Spherical Cancer Models in Tumor Biology. **Neoplasia (United States)**, v. 17, n. 1, p. 1–15, 2015. Disponível em: <http://dx.doi.org/10.1016/j.neo.2014.12.004>.
WILCZYNSKA, A.; BUSHELL, M. The complexity of miRNA-mediated repression. **Cell Death and Differentiation**, v. 22, n. 1, p. 22–33, 2015. Disponível em: <http://dx.doi.org/10.1038/cdd.2014.112>.
WITTENBURG, L. A. et al. The histone deacetylase inhibitor valproic acid sensitizes human and canine osteosarcoma to doxorubicin. **Cancer Chemotherapy and Pharmacology**, v. 67, n. 1, p. 83–92, 2011.
WITTENBURG, L. A.; GUSTAFSON, D. L.; THAMM, D. H. Phase I pharmacokinetic and pharmacodynamic evaluation of combined valproic acid/doxorubicin treatment in dogs with spontaneous cancer. **Clinical Cancer Research**, v. 16, n. 19, p. 4832–4842, 2010.
WU, Q. et al. A chemical toolbox for the study of bromodomains and epigenetic signaling. **Nature Communications**, v. 10, n. 1, p. 1–14, 2019. Disponível em: <http://dx.doi.org/10.1038/s41467-019-09672-2>.
WUCHER, V. et al. FEELnc: A tool for long non-coding RNA annotation and its application to the dog transcriptome. **Nucleic Acids Research**, v. 45, n. 8, p. 1–12, 2017.
XAVIER, P. L. P. et al. An epigenetic screening determines BET proteins as targets to suppress self-renewal and tumorigenicity in canine mammary cancer cells. **Scientific Reports**, v. 9, n. 1, p. 1–15, 2019.
YAMAZAKI, J. et al. Dynamic changes in DNA methylation patterns in canine

lymphoma cell lines demonstrated by genome-wide quantitative DNA methylation analysis. **Veterinary Journal**, v. 231, p. 48–54, 2018. Disponível em: <http://dx.doi.org/10.1016/j.tvjl.2017.11.007>.
YANG, C. M. et al. Aberrant DNA hypermethylation-silenced SOX21-AS1 gene expression and its clinical importance in oral cancer. **Clinical epigenetics**, v. 8, p. 129, 2016. Disponível em: <http://dx.doi.org/10.1186/s13148-016-0291-5>.
YANG, X. et al. Gene body methylation can alter gene expression and is a therapeutic target in cancer. **Cancer Cell**, v. 26, n. 4, p. 577–590, 2014. Disponível em: <http://dx.doi.org/10.1016/j.ccr.2014.07.028>.
YANG, X.; WONG, M. P. M.; NG, R. K. Aberrant DNA methylation in acute myeloid leukemia and its clinical implications. **International Journal of Molecular Sciences**, v. 20, n. 18, 2019.
YOO, C.; CHENG, J.; JONES, P. A. Zebularine: A new drug for epigenetic therapy. **Biochemical Society transactions**, v. 32, p. 910–912, 1 jan. 2005.
YU, R. M. C.; CHEAH, Y. K. The roles of miRNAs in human breast cancer and canine mammary tumor. **Applied Cancer Research**, v. 37, n. 1, p. 1–11, 2017.
ZANINI, D. A. et al. Environmental risk factors related to the development of canine non-Hodgkin's lymphoma. **Ciência Rural**, v. 43, n. 7, p. 1302–1308, 2013.
ZHANG, Y. et al. Analysis of the androgen receptor–regulated lncRNA landscape identifies a role for ARLNC1 in prostate cancer progression. **Nature Genetics**, v. 50, n. 6, p. 814–824, 2018. Disponível em: <https://doi.org/10.1038/s41588-018-0120-1>.
ZHANG, Z. et al. Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast. **Breast Cancer Research and Treatment**, v. 94, n. 1, p. 11–16, 2005.
ZHOU, S. S. et al. MiRNAS in cardiovascular diseases: Potential biomarkers, therapeutic targets and challenges review-article. **Acta Pharmacologica Sinica**, v. 39, n. 7, p. 1073–1084, 2018.
ZORZAN, E. et al. Mutational hotspot of TET2, IDH1, IDH2, SRSF2, SF3B1, KRAS, and NRAS from human systemic mastocytosis are not conserved in canine mast cell tumors. **PLoS ONE**, v. 10, n. 11, p. 1–11, 2015.

3. Chapter 2: ZEB1/2 Transcription Factors Are Potential Therapeutic Targets of Canine Mammary Cancer Cell Lines

Pedro L. P. Xavier [1]; Yonara G. Cordeiro [1]; Arina L. Rochetti [1]; Juliano R. Sangalli [2]; Debora A.P.C Zuccari [3]; Juliano C. Silveira [2]; Fabiana F. Bressan [2]; Heidge Fukumasu[1]

[1] Laboratory of Comparative and Translational Oncology (LOCT), Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil

[2] Laboratory of Molecular Morphophysiology and Development (LMMD), Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil

[3] Laboratory of Molecular Investigation of Cancer (LIMC), Faculty of Medicine of São Jose do Rio Preto (FAMERP), São Jose do Rio Preto, Brazil

Published in Veterinary and Comparative Oncology 16(4):596-605. Doi:10.1111/vco.12427. Jul 2018. Analysis and discussion regarding the transcriptomic data from this chapter are not included in the original article published.

Abstract

Mammary tumours are the most frequent in female dogs as in women and half are malignant. Tumorigenicity and invasiveness are important acquired characteristics for the development and progression of cancers and could be regulated by transcription factors associated with epithelial-mesenchymal transition (EMT) as ZEB1, ZEB2, SNAI1, SLUG and STAT3. Thus, here, we evaluated the expression of EMT-associated transcription factors in canine mammary cancer (CMC) cell lines characterized for invasiveness and tumorigenicity to determine if these could be considered good targets for future development of therapies. Furthermore, we evaluated the transcriptomic profile of mesenchymal-like cancer cell lines (ME cells) in comparison with one epithelial-like cancer cell line (EP cells). Five CMC cell lines were characterized regarding their morphology, doubling time and expression of intermediate and actin filaments. In addition, gene expression of *SLUG*, *STAT3*, *ZEB1*, *ZEB2* and *CDH1*, tumorigenicity and invasiveness were assessed. Two of these cells presented an epithelial-like morphology (E20 and E37) and three a mesenchymal-like morphology (M5, M25 and CF41.Mg). M25 and CF41.Mg presented higher invasiveness. Furthermore, only ME cells formed

tumorspheres and CF41.Mg made more and larger tumorspheres. The ME cells were more malignant than the EP cells being the CF41.Mg the most malignant. CF41.Mg presented higher *ZEB1* and *ZEB2* and lower *CDH1* gene expression. Furthermore, our results revealed that there is a positive correlation between *ZEBs* and the tumorsphere number and size. Finally, transcriptomic and functional enrichment analysis showed that upregulated genes in ME cells were related to important KEGG and Reactome pathways associated with EMT, invasiveness and tumorigenicity including ECM organization and degradation, focal adhesion, TGF-β/BMP signaling, PI3K-AKT signaling, WNT signaling and regulation of insulin-like IGF transport and uptake by IGF binding proteins. In conclusion, these findings support ZEB1 and ZEB2 as potential therapeutic targets for CMC cells. In addition, we identified that upregulated differentially expressed genes in the ME cells are enriched to key processes associated with tumorigenicity and invasiveness.

Keywords: canine mammary cancer, comparative oncology, EMT-associated transcription factors, invasion potential, tumorigenicity

3.1 Introduction

Mammary gland tumors are the most frequent tumors in female dogs as in women, corresponding to 52% of all types of tumors, in which half of those are malignant (HELLMÉN, 2005; SLEECKX et al., 2011; SALAS et al., 2015). Mammary tumors in both species have similar biological features; thus, canine cancer and cell lines are excellent experimental models for comparative development of new therapies, identification of cancer-associated genes and pathways and understanding of cancer biology and progression (PAOLONI; KHANNA, 2008).

Tumorigenicity and invasiveness are important acquired characteristics for the development and progression of cancer. The first is determined by the ability of cancer cells to generate new tumors (TABASSUM; POLYAK, 2015), while the second is the capacity to invade the extracellular matrix (ECM) in order to disseminate through the blood or lymphatic vessels and colonize a secondary tissue, generating metastasis (VALASTYAN; WEINBERG, 2011). Recently, our group showed that the overexpression of genes involved in focal adhesion and extracellular matrix communication (*FN1*, *ITGA8* and *THBS2*) is associated with invasiveness and *in vivo* tumorigenesis of canine mammary cancer (CMC) cell lines (CORDEIRO et al., 2018).

However, the identification of master regulators of these phenotypes is crucial to develop novel therapeutic targets.

In fact, there are evidences that epithelial to mesenchymal transition (EMT)-associated transcription factors including *ZEB1*, *ZEB2*, *SNAI1*, *SLUG* and *STAT3* are important in the regulation of tumorigenicity and invasiveness in human cancer cells (WELLNER et al., 2009; SMITH et al., 2014; QIAO et al., 2015; LUANPITPONG et al., 2016). One study showed higher ZEB1 expression in the canine mammary tumor and its derived cell-line in comparison to the normal mammary tissue, displaying an EMT phenotype mediated by TGF-β/ZEB1/miR-200 regulatory loop (RAPOSO et al., 2017). Another study showed that ZEB2 expression predicted a poor survival rate in invasive micropapillary carcinoma of the mammary gland (IMPC) while ZEB1 was associated with the differentiation process of these IMPC (GAMBA et al., 2014). However, ZEB1 appeared to not exert E-cadherin transcription repression activity in lymph node metastasis from IMPC (GAMBA et al., 2015). Taken together, these works still did not evidenced the potential of these transcriptional factors as targets for new therapies for canine mammary tumors.

Therefore, the aim of this study was to evaluate the expression of EMT-associated transcription factors in canine mammary cancer cell lines characterized for invasiveness and tumorigenicity to determine if these could be considered for the future development of target therapies. In addition, we also evaluated the transcriptomic profile of these cell lines to determined potential molecular pathways associated with invasiveness and tumorigenicity in canine mesenchymal-like cancer cells.

3.2. Material and Methods

3.2.1. Canine mammary cancer cell lines and cell culture

Five CMC cell lines were used in this study, where four were isolated and characterized in our laboratory, as previously described (CORDEIRO et al., 2018). Two of these cells presented an epithelial-like morphology (EP cells) (E20 and E37) and two a mesenchymal-like morphology (ME cells) (M5 and M25). These cells were cultured for 25 passages to obtain a uniform and clonal cell population. Also, the commercially available CF41.Mg cell line was kindly donated by Dr. Debora A. P. C. Zuccari (Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, São Paulo,

Brazil). All cell lines were maintained in 75 cm^2 flasks at 37ºC and 5% CO_2 with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Pen-strep). Passaging were performed when cells were 85% confluent using TrypLE™ Express trypsin. Culture evolution was evaluated daily by optical microscopy (Axio Vert A1, Zeiss, Germany). All reagents used for cell culture were purchased from Thermo Fisher Scientific, USA.

3.2.2. Doubling time

For doubling time assay, 5 x 10^4 cells were plated in 6-well plates (Corning, USA), supplemented with culture medium as described above. The number of cells was counted using a hemocytometer at 24-hour intervals for a total period of 96 hours. The results were evaluated with the Doubling-Time Software (http://www.doubling-time.com/compute_more.php).

3.2.3. Immunocytochemistry of intermediate filaments

The immunocytochemistry was performed in cells growing in a 6-well plate (Corning, USA) containing 2 ml of supplemented medium and cultured up to 80% confluence. Then, cells were rinsed and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Cells were rinsed twice and blocked with 5% milk in PBS for 30 minutes at RT and later were incubated with specific primary antibodies to cytokeratin (clone A1/A3, Dako, USA) and vimentin (clone V9, Dako, USA) diluted in PBS (1:100) at 4ºC overnight. Cells were rinsed three times and incubated at RT for 1 hour with the corresponding Alexa Fluor® 488 Goat Anti-mouse IgG (H+L) secondary antibody at a dilution of 1:250. Finally, cells were incubated in Hoechst 33342 for 10 minutes at RT following manufacturer's recommendations (Thermo Fisher, USA). All washes were done using with the blocking solution, containing PBS with 0.1% BSA and tween 0.1%. Cells were observed for immunofluorescence using a ZEISS - Axio Vert A1 with a camera Axio Can 503 attached using a 520 nm and 455 wavelength filters for green and blue color, respectively (ZEISS, Germany). Pictures were taken using ZEISS ZEN 2 Microscope Software.

3.2.4. Detection of actin filaments

To assess the actin filaments, cells were seeded in a 96-well plate (Corning, USA). Then, at 80% confluency cells were rinsed and fixed with 4% paraformaldehyde for 30

minutes at room temperature, rinsed three times with PBS and incubated in Phalloidin-Atto 565 diluted in PBS (1:200) for 30 minutes at RT. Cells were rinsed three times and incubated in DAPI for 15 minutes at RT. Finally, cells were rinsed and observed for immunofluorescence using a ZEISS - Axio Vert A1 with a camera Axio Can 503 attached using a 620 nm and 455 nm wavelength filter for red and blue color, respectively (ZEISS, Germany). Pictures were taken using ZEISS ZEN 2 Microscope Software (ZEISS, Germany).

3.2.5. Invasion assay

Cells were cultured for 24 hours in serum-free medium. The transwell inserts were placed in 24-well plates and filled with 100 µl of ECM gel (Sigma Aldrich®, USA) in PBS (1:5). After, 4 x 10^4 cells were resuspended in 100 µl serum-free medium and plated on inserts. The bottom well was filled with 600 µl of DMEM-F12 medium supplemented with 20% FBS, used as chemoattractive, and 24 hours later, a cotton swab was used to remove non-invasive cells from the top of the inserts. As fixative, 5% glutaraldehyde was used for 10 minutes at room temperature and inserts stained with 1% crystal violet in 2% ethanol for 2 minutes. The invasive cells were observed and photographed under an optical microscope in five random fields at 100x magnification using ZEISS ZEN 2 Microscope Software (ZEISS, Germany). Finally, the invasive cells were counted using ImageJ software (SCHNEIDER; RASBAND; ELICEIRI, 2012) . The experiment was performed twice and in duplicates.

3.2.6. Tumorsphere formation assay

The tumorsphere formation ability was evaluated using an ultra-low attachment surface 24-well plate (Corning, USA). One thousand cells were plated per well containing 0.5 mL of serum-free medium supplemented with 20 ng/ml of EGF (PrepoTech, USA), 10 ng/ml of FGF (PrepoTech, USA) and 5µl/ml of bovine insulin (Sigma Aldrich, USA). Tumorspheres number and size were evaluated 7 days after seeding. For measuring analysis, 10 tumorspheres per replicate were photographed and analyzed using ZEISS ZEN 2 Microscope Software (ZEISS, Germany).

3.2.7. RNA extraction, cDNA synthesis and real time PCR

Cells were cultured as described in 75cm^2 flasks and when reached 85% confluency total RNA was extracted using Trizol® following the manufacturer's

instructions. RNA samples were quantified and the 260/280 and 260/230 ratio (**Supplementary Table S1, Appendix B**) was determined by NanoDrop 2000^{TM} (Thermo Fisher Scientific, USA). Only samples which presented values between 1.6-2.1 and demonstrated good quality (not degraded) after the electrophoretic analysis in agarose gel (1% TAE) were used. Thus, cDNA was synthesized from 1 µg of total RNA with oligoDT primers using the High Capacity cDNA Reverse Transcription kit according to manufacturer's protocol. All the reagents used in this experiment, were purchased from Thermo Fisher Scientific, California, USA.

Gene expression analysis were performed by real time quantitative PCR using a StepOne System (Thermo Fisher Scientific, USA). Specific primers for each gene were designed with Primer-BLAST (YE et al., 2012) and the possibility of dimers and hairpins was verified using AutoDimer software (VALLONE; BUTLER, 2004). Primers were also analyzed by in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) to confirm specificity. Primers sequences are reported in the **Supplementary Table S2, Appendix B**. All PCR reactions were carried out using Fast SYBR Green Master Mix in a final volume of 10 µl. Conditions for quantitative PCR were as follows: 95ºC for 20 s; 40 cycles at 95ºC for 3 s for denaturation, 60ºC for 30 s for anneal/extend; melt curve analysis was performed at 95ºC for 15 s and 60ºC for 60 s. The housekeeping gene used was the 18s ribosomal RNA and the analysis of relative gene expression data was performed according to the 2-Delta-Delta-CT method (LIVAK; SCHMITTGEN, 2001). The experiment was performed twice and in biological triplicates.

3.2.8 Western blotting analysis

Protein lysates were obtained from all cell cultures using RIPA buffer, mixed with Laemmli buffer (4x) (Bio-Rad, CA, USA), boiled at 95ºC for 5 min, and then loaded onto an 8%-SDS-PAGE. Electrophoresis was performed at 100 V constant for 2 hours and the proteins were transferred to a nitrocellulose membrane in a wet transfer apparatus for 2 hours at 80 V constant. The membranes were then placed in a blocking buffer containing 5% bovine serum albumin (BSA) in tris-buffered saline with 0.1% tween (TBST) for 1 h at room temperature. Then, the membranes were incubated overnight at 4 °C with the anti-ZEB1 rabbit antibody (1:500, Sigma Aldrich, USA), and anti-E-cadherin rabbit antibody (1:1000, Cell Signaling Technology, USA). After this, the membranes were

washed thrice for 5 min with TBST and then incubated with the secondary antibody, anti-rabbit conjugated with HRP antibody (1:5000, Sigma Aldrich, USA), for 1h at room temperature. The membranes were washed thrice in 1x TBST for 5min. For detection, the membranes were subjected to a chemiluminescent reaction using the reagent Clarity Western ECL substrate (Bio Rad, Hercules, CA, USA). Imaging and band density analyses were performed using ChemiDoc MP Image System (Bio-Rad, USA) and the software Image Lab 5.1, respectively. The relative amounts of ZEB1 and E-cadherin were normalized using the monoclonal Anti-β-Actin−Peroxidase antibody produced in mouse (1:100,000 cat #A3854, Sigma) blotted in the same membranes. The experiment was performed in biological triplicates.

3.2.9 RNA-seq Data Generation (This session is not included in the original article)

Total RNA of canine mammary cancer cells was extracted using Trizol® following the manufacturer's instructions. The RNA quality and quantity were assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to the manufacturer's instructions (Agilent Technologies, Ireland). Only samples that presented an RNA integrity number (RIN) higher than 8.0 were considered to the sequencing. RNA libraries were constructed using the TruSeq™ Stranded mRNA LT Sample Prep Protocol and sequenced on Illumina HiSeq 2500 equipment in a HiSeq Flow Cell v4 using HiSeq SBS Kit v4 (2x100pb).

3.2.10 Alignment and Differential Expression (This session is not included in the original article)

Sequencing quality was evaluated using the software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and no additional filter was performed. Sequence alignment against the canine reference genome (CanFam3.1) was performed using STAR (DOBIN et al., 2013), according to the standard parameters and including the annotation file (Ensembl release 89). Secondary alignments, duplicated reads and reads failing vendor quality checks were removed using Samtools (LI et al., 2009). Alignment quality was confirmed using Qualimap (GARCÍA-ALCALDE et al., 2012). Gene expression was estimated by read counts using HTseq (ANDERS; PYL; HUBER, 2015) and normalized as counts per million reads (CPM). Only genes presenting at least 1 CPM were kept for differential expression (DE) analysis. DE was performed using EdgeR package (ROBINSON; MCCARTHY; SMYTH, 2009) on R environment,

based on negative binomial distribution. Benjamini-Hochberg procedure was used to control the false discovery rate (FDR) and transcripts presenting FDR ≤ 0.01 and log-fold change (LogFC) >1; <1 were considered differential expressed (DE). Functional enrichment analysis of DE genes was performed using STRING (JENSEN et al., 2009; SZKLARCZYK et al., 2019).

3.2.11 Statistical analysis

Statistical analysis and graphics were made with GraphPad Prism® 6.0 software (San Diego, CA, USA). Gene expression, invasion assay and tumorsphere formation were analyzed by one-way ANOVA with posthoc Tukey. Correlation analyses were calculated with Spearman test. Data are presented as mean ± standard deviation unless otherwise indicated. Significant differences were considered when $p < 0.05$.

3.3 Results

3.3.1. Morphological characterization of canine mammary cancer cell lines

First, the five cell lines were analyzed morphologically and by expression of intermediate filaments. Four cell lines were previously established in our lab showing a mesenchymal-like (ME) (M5 and M25) (CORDEIRO et al., 2018) or epithelial-like (EP) phenotype (E20 and E37). In addition, a commercial established cell line (CF41.Mg) was used in this work showing a ME morphology (**Figure 1**). Doubling time of these cell lines ranged from 14.74h to 42.8h (**Table 1**).

Table 1. Morphological characterization of five canine cancer cell lines

Cell line	**Cytokeratin**	**Vimentin**	***E-cad***	**Doubling time (h)**	**Morphology type**	**Tumor of origin**
E20	++	+	+++	20.37	Polygonal	Mixed Carcinoma
E37	+++	+++	+	14.74	Polygonal	Mixed Mammary Tumor
M5	+	++	-	26.0	Spindle	Comedocarcinoma
M25	+	+	-	42.8	Spindle	Mixed Carcinoma
CF41.Mg	-	++	-	20.25	Spindle	Carcinoma

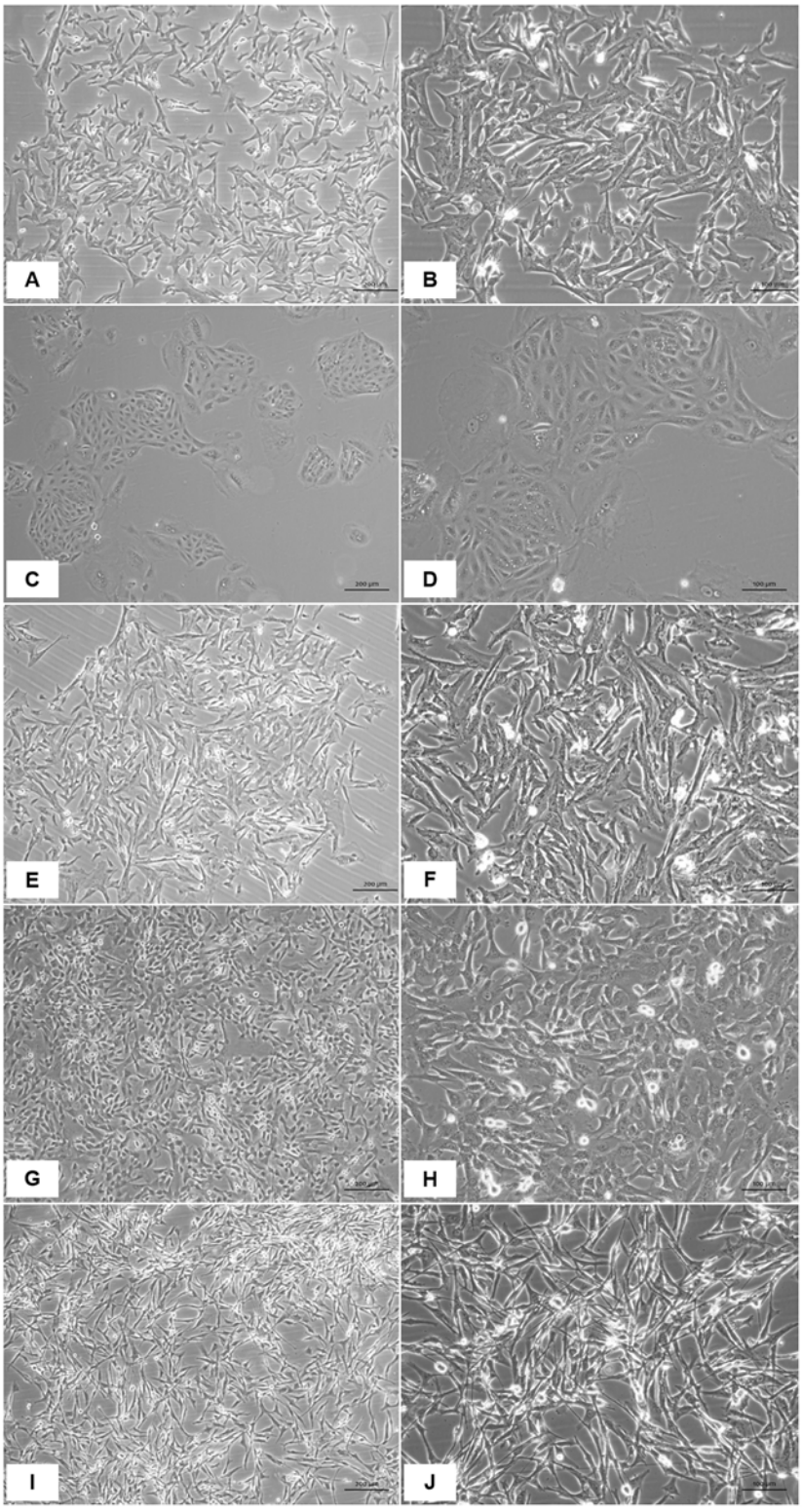


Figure 1. Morphology of five canine mammary cancer cell lines. The E20 (**C-D)** and E37 (**G-H**) cell lines exhibited epithelial-like morphology, with polygonal shape, juxtaposed cells groups and little intracellular space. The M5 (**A-B**), M25 (**E-F**) e CF41.Mg (**I-J**) showed mesenchymal-like morphology, with fusiform and scarce cytoplasm. The images were obtained by optical microscopy using 5X and 10X objectives.

Immunofluorescence analysis of intermediate filaments showed EP cell lines (E20 and E37 cells) expressing high cytokeratin. The E37 cells exhibited a homogeneous expression of both proteins but the vimentin expression was distributed in a diffuse way in E20 cells (**Table 1** and **Figure 2**). On the other hand, the ME cell line CF41.Mg showed high expression of vimentin but no expression of cytokeratin (**Figure 2**). In addition, we found that E20 and E37 cells presented E-cadherin expression, an adhesion protein considered an epithelial marker (**Table 1**). Regarding the actin filaments, all cell cultures presented high density and disorganized arrangement. Also, projections of actin filaments such as filopodia (**Figure 2**), which are typical of invasive cancer cells, were observed mainly in ME cells. Therefore, we have classified the five CMC cell lines into epithelial- or mesenchymal-like based on morphology and expression of cytokeratin, vimentin, and e-cadherin (**Table 1**).

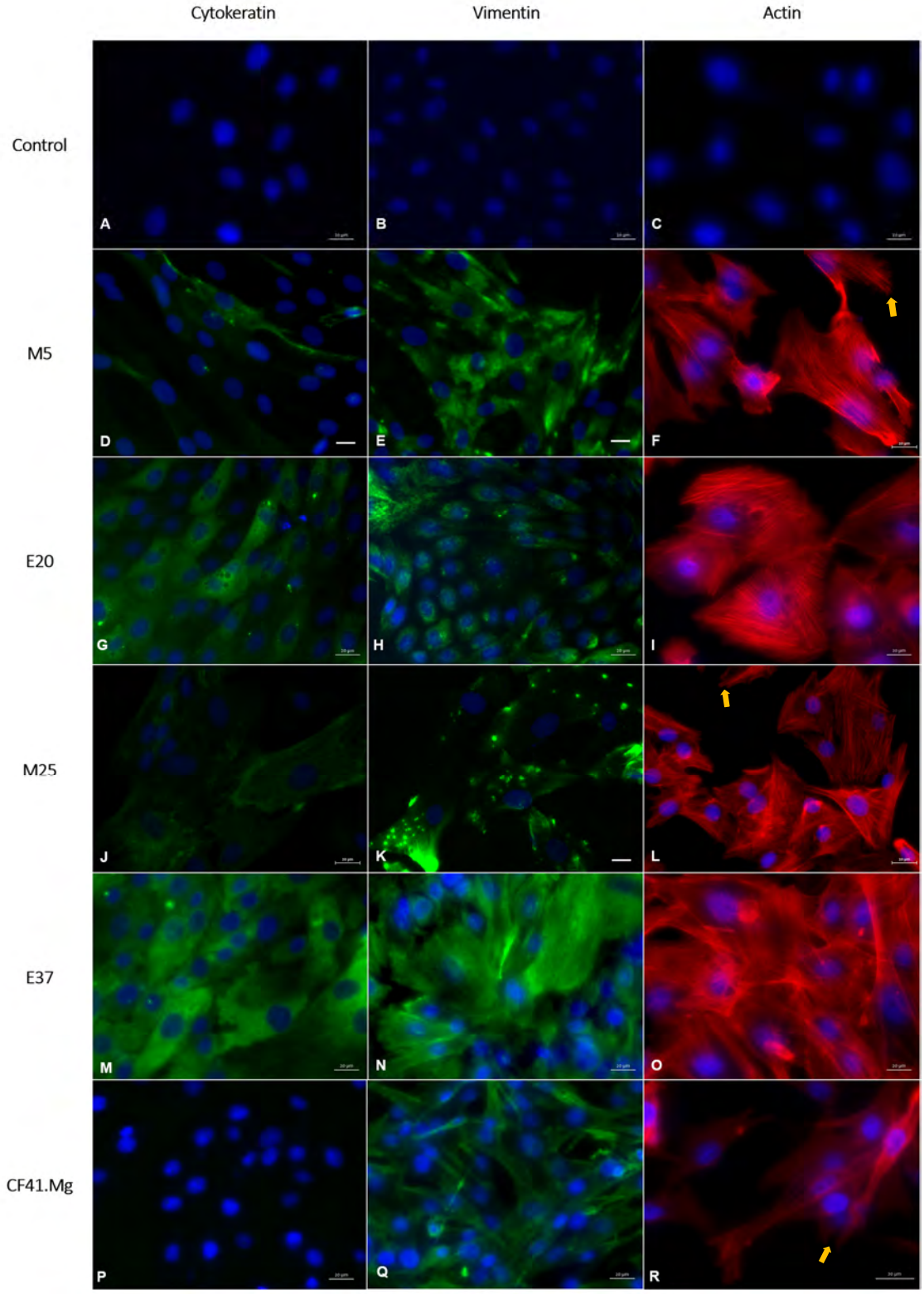


Figure 2. Intermediate filaments expression in canine mammary cancer cell lines. Figures **A, B and C** represent the negative controls to cytokeratin, vimentin, and actin, respectively. The nucleus is staining with blue color. The E20 (**G-H)** and E37 (**M-N**) cells exhibited similar expression of cytokeratin (Green) and vimentin (Green), where the vimentin expression was distributed diffusely in E20 cells. E37 cells exhibit a homogeneous expression of both cytokeratin and vimentin. The M5 (**D-E**), M25 (**J-K**)

and CF41.Mg cells (**P-Q**) showed higher vimentin expression and low cytokeratin expression. All cell lines showed disorganized arrangement of actin (red) filaments (**F-I-L-O-R**) and ME cells exhibited projections such as filopodia (yellow arrows), typical of malignant and invasive cells. The images were obtained using 40x objective.

3.3.2 Invasiveness and tumorigenicity of canine mammary cancer cell lines

All CMC cell lines were positive in the *in vitro* invasion assay, where two of three ME cells (M25 and CF41.Mg) were significantly more invasive than the others ($p < 0.0001$; **Figure 3**). The CF41.Mg cell line presented the highest potential for degrading the ECM gel and invade the transwell insert.

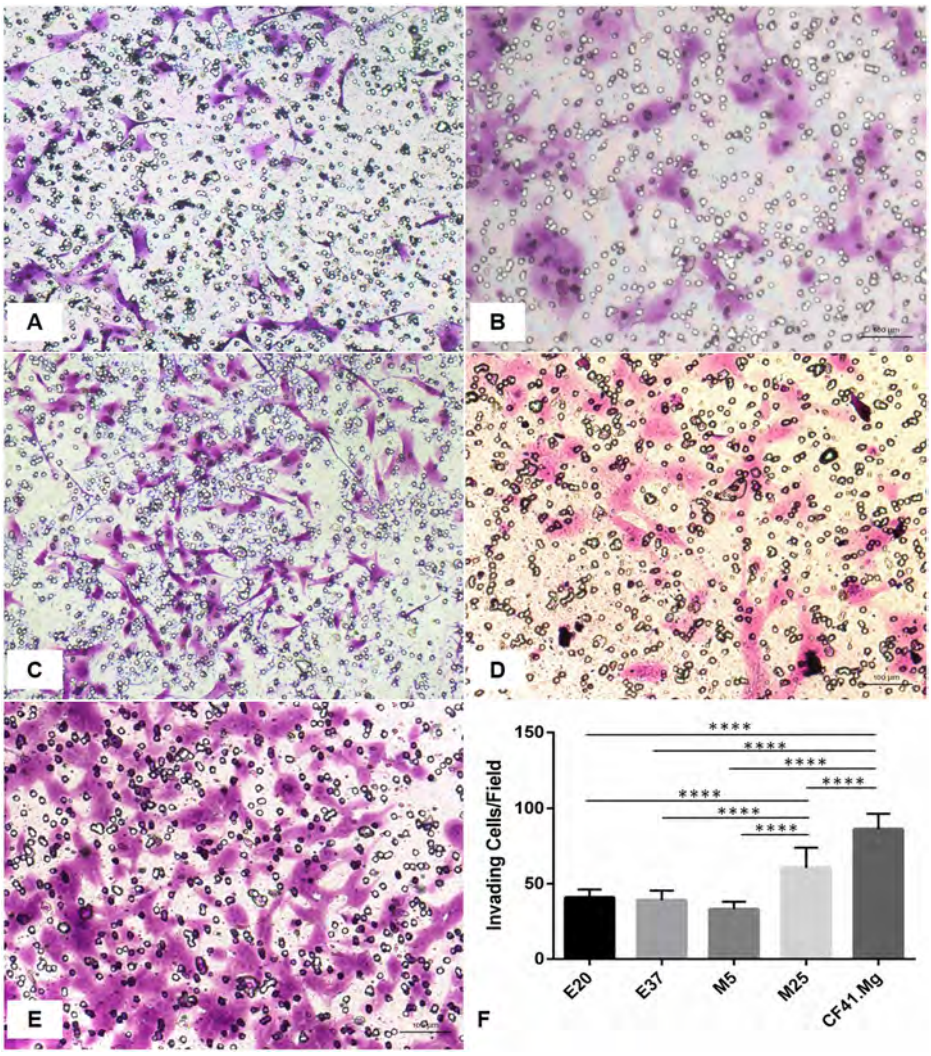


Figure 3. *In vitro* invasion assay of canine mammary cancer cell lines. M5 (**A**), E20 (**B**), M25 (**C**), E37 (**D**) and CF41.Mg (**E**) cells were positive for the *In vitro* invasion assay. CF41.Mg cells showed higher invasive potential in comparison to the other cell lines (**E,F**). The images were obtained in a 10x objective. (****$p < 0.0001$ – One-way ANOVA followed by Tukey's multiple comparison test).

Under anchorage-independent conditions, M5, M25 and CF41.Mg cells generate tumorspheres whereas the EP cells E20 and E37 didn't. Between the ME cells, the CF41.Mg and M5 cells formed more tumorspheres than M25 ($p < 0.0001$; **Figure 4**). In addition, the tumorspheres from CF41.Mg cells were significant larger ($p < 0.0001$; **Figure 4**).

Taken together, these results demonstrated that CF41.Mg cell line was more invasive and formed more and bigger tumorspheres. Therefore, the ME cells were more malignant than EP cells and the CF41.Mg cell line was considered the most malignant.

3.3.3 Expression of EMT-associated transcription factors in canine mammary cancer cell lines

Four EMT-associated transcription factors were found expressed in all cell lines (SLUG, STAT3, ZEB1 and ZEB2; **Figure 5**) but only *ZEB1* and *ZEB2* were found to be positively associated with tumorsphere number ($r = 0.9747$; $p < 0.05$; **Table 2**) and size ($r = 0.9747$; $p < 0.05$; **Table 2**). Interestingly, CF41.Mg cell line significantly expressed more *ZEB1*, *ZEB2* and less *SLUG* than the other cell lines (**Figure 5** and **Figure 6**).

No difference was observed on *STAT3* gene expression. In addition, *CDH1* gene expression in canine mammary cancer cells was inversely correlated with *ZEB1/ZEB2* ($r = -0.9$, $p < 0.05$; **Table 3**) but not with SLUG and STAT3 (**Table 3**). Western Blotting analysis validated the gene expression analysis for ZEB1 and E-cadherin and their inverse correlation ($r = -0.8944$, $p < 0.0001$; **Figure 6**). We also observed a positive correlation between *ZEB1* and *ZEB2* at mRNA level ($r = \sim1$; $p = 0.0167$; **Table 3**). At last, our results revealed that there is a positive correlation between *ZEB1/ZEB2* expression and the tumorsphere number ($r = 0.9747$; $p = 0.0333$; **Table 2**) and size ($r = 0.9747$; $p = 0.0333$; **Table 2**).

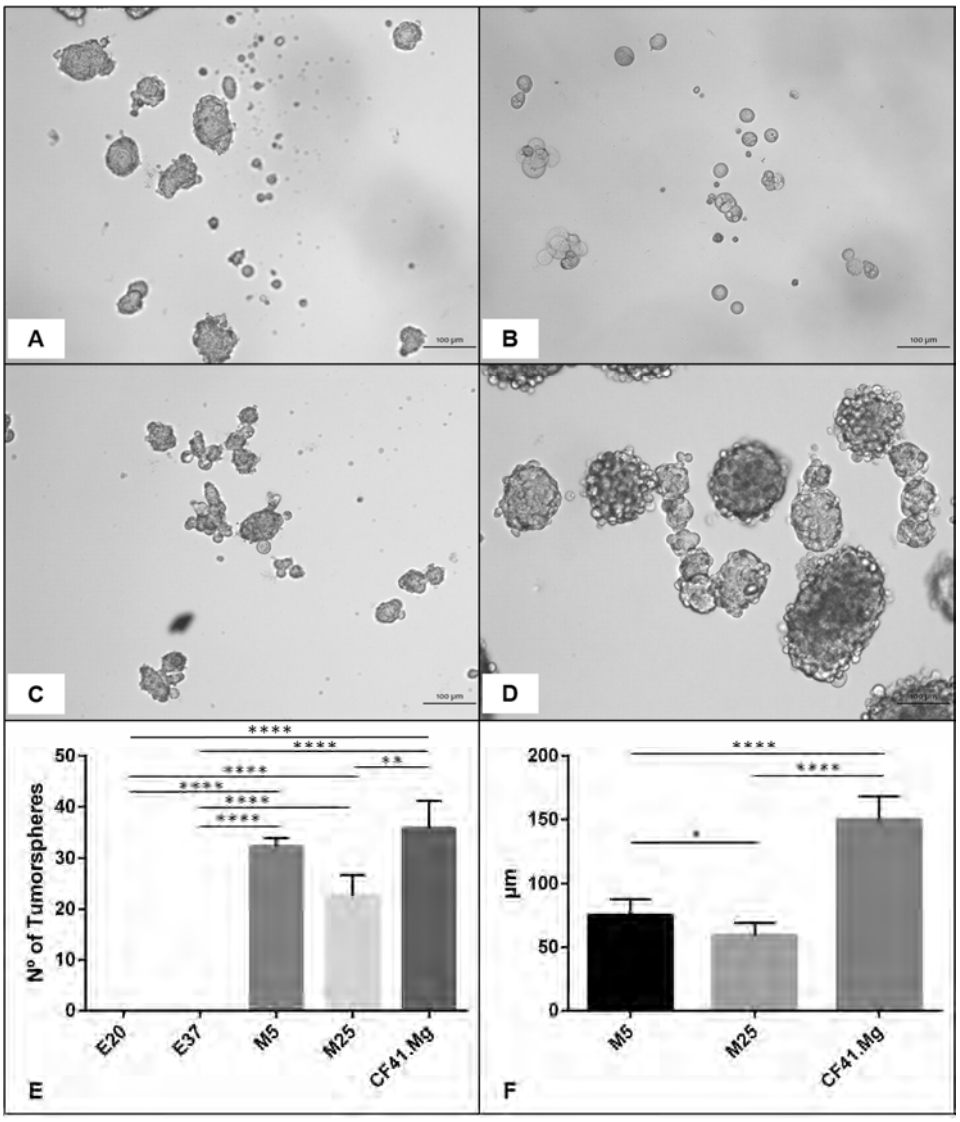


Figure 4. **Tumorsphere formation potential of CMC cell lines**. The M5 (**A**), M25 (**C**) and CF41.Mg (**D**) were able to form tumorspheres where M5 and CF41.Mg presented higher tumorsphere formation potential (**E**). In addition, CF41.Mg cells exhibited larger tumorspheres (**F**). E20 (**B**) and E37 cells (**Supplementary Figure S1, Appendix B**) were not able to generate tumorspheres. The images were obtained in a 10x objective. (*p < 0.05; **p < 0.01; ****p < 0.0001 – One-way ANOVA followed by Tukey's multiple comparison test).

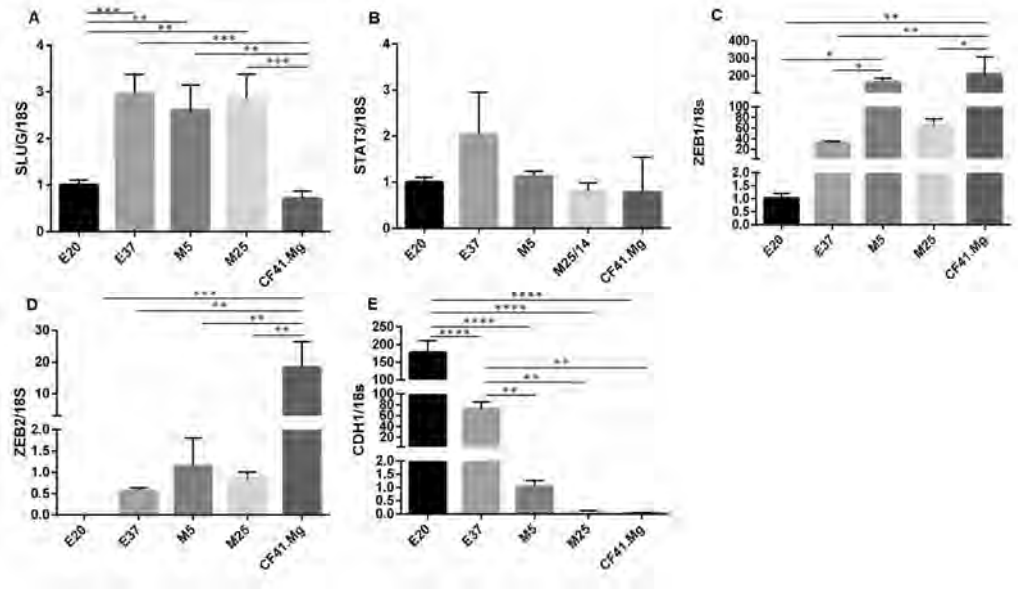


Figure 5. Gene expression of EMT-associated Transcription Factors and E-cadherin (*CDH1*). The E37, M5 and M25 cells presented the higher levels of *SLUG* expression (**A**). M5 and CF41.Mg cells showed the highest levels of *ZEB1* expression (**C**). Still, CF41.Mg presented the highest *ZEB2* expression (**D**). Finally, *CDH1* expression was highly expressed in E20 and E37 epithelial cells (**E**), showed an inverse correlation with *ZEB1* and *ZEB2* (p = 0.0417). There was no statistical difference regarding *STAT3* gene expression (**B**) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p <$ 0.0001 - One-way ANOVA followed by Tukey's multiple comparison test).

Table 2. Correlation between transcription factors and *CDH1* expression and tumorspheres number and size.

Genes	Tumorsphere number	*p* Value	Tumorsphere size	*p* Value
SLUG	-0.5643	0.15	-0.5643	0.3
STAT3	-0.6156	0.13	-0.6156	0.2667
ZEB1	0.9747	0.0167	0.9747	0.0333
ZEB2	0.9747	0.0167	0.9747	0.0333
CDH1	- 0.8721	0.0167	-0.8721	0.0333

Data are presented as r value. Significant differences were considered when $p < 0.05$.

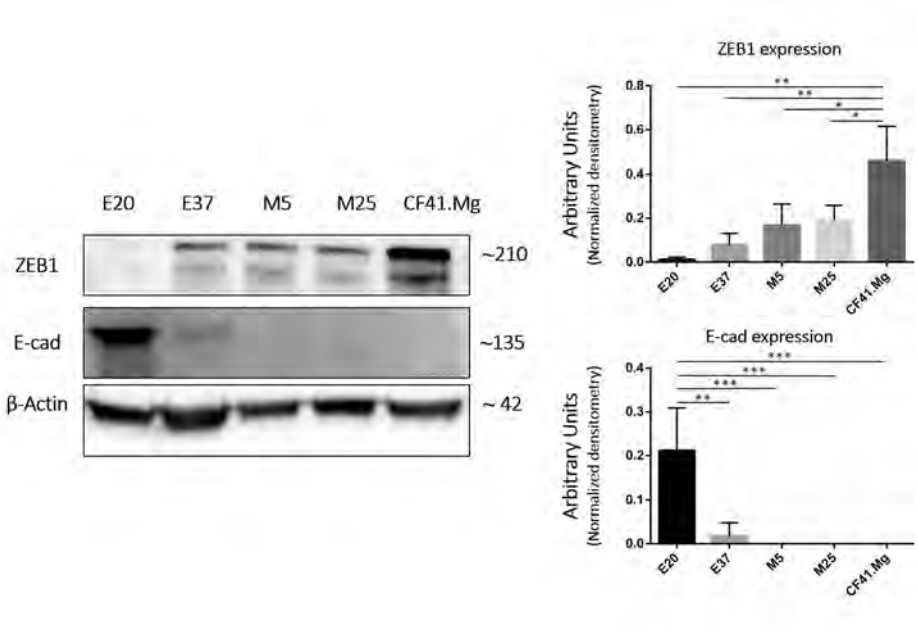


Figure 6. ZEB1 and E-cadherin protein levels from CMC cell lines. CF41.Mg cells presented the higher levels of ZEB1 protein. Only epithelial-like cells E20 and E37 expressed E-cadherin. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ – One-way ANOVA followed by Tukey's multiple comparison test).

Table 3. Correlation between transcription factors and *CDH1* expression.

Genes	***SLUG***	***STAT3***	***ZEB1***	***ZEB2***	***CDH1***
SLUG		0.7	-0.4	-0.4	0.3
STAT3	0.7		-0.5	-0.5	0.7
ZEB1	- 0.4	0.5		~ 1**	-0.9*
ZEB2	-0.4	-0.5	~ 1**		-0.9*
CDH1	0.3	0.7	-0.9*	-0.9*	

Data are presented as r value. * $p < 0.05$, ** $p < 0.01$

3.3.4 Transcriptomic analysis of ME cells and EP cells (These data are not included in the original article)

To perform this analysis, we classified the canine mammary cancer cells in two different groups: 1) cells able to generate tumorspheres (ME cells M5, M25 and CF41.Mg) and 2) cells unable to generate tumorspheres (E20). An average of 30 million paired-end reads were sequenced per sample and an average of 88% were aligned to the reference genome as concordant pairs (**Supplementary Table S3, Appendix B**). A total of 13.555 genes passed quality control and were tested for differential expression (DE). Of these 1.142 genes were upregulated and 1.069 were downregulated in the ME cells in comparison with E20 cell line (FDR< 0.01 and LogFC > 1; < -1). Functional enrichment analysis showed that upregulated genes in ME cells were related to KEGG and Reactome pathways including important pathways associated to EMT, invasiveness and tumorigenicity such as TGF-β signaling, PI3K-AKT signaling, proteoglycans in cancer, pathways in cancer, WNT signaling, extracellular matrix organization, collagen formation, ECM-receptor interaction, ECM proteoglycans, degradation of the extracellular matrix, regulation of insulin-like IGF transport and uptake by IGF binding proteins, signaling by BMP and signaling by receptor tyrosine kinases (**Table 4**). Downregulated genes in ME cells were enriched to pathways including antigen processing and presentation, RIG-I-like receptor signaling, cell adhesion molecules, metabolism of xenobiotics by cytochrome P450, NF-kappa B signaling, immune system, cell junction organization, cell-cell communication, signaling by receptor tyrosine kinases, adaptative immune system, innate immune system, cytokine signaling in immune system, ISG15 antiviral mechanism, and Interferon Signaling (**Table 5**).

Table 4. KEGG and Reactome pathway analysis of upregulated genes in ME cell lines in comparison to EP cell line.

KEGG Pathways – upregulated genes in mesenchymal-like cell lines			
Pathway	**Description**	**Count in gene set**	**FDR**
cfa04512	ECM-receptor interaction	29 of 76	4.12e-08
cfa04510	Focal adhesion	44 of 184	1.20e-07
cfa04151	PI3K-Akt signaling pathway	55 of 319	1.24e-05
cfa05205	Proteoglycans in cancer	32 of 183	0.0015
cfa05200	Pathways in cancer	63 of 475	0.0015
cfa04350	TGF-β signaling pathway	17 of 72	0.0026
cfa04024	cAMP signaling pathway	30 of 183	0.0040
cfa04015	Rap1 signaling pathway	31 of 193	0.0042
cfa04933	AGE-RAGE signaling pathway in diabetic complications	19 of 93	0.0046
cfa04310	WNT signaling pathway	22 of 128	0.0110
cfa05217	Basal cell carcinoma	13 of 60	0.0187
cfa04810	Regulation of actin cytoskeleton	28 of 197	0.0263
cfa04922	Glucagon signaling pathway	16 of 89	0.0271
cfa04390	Hippo signaling pathway	21 of 137	0.0354
Reactome Pathways – upregulated genes in mesenchymal-like cell lines			
CFA-1474244	Extracellular matrix organization	83 of 253	3.79e-21
CFA-1650814	Collagen biosynthesis and modifying enzymes	29 of 63	1.84e-09
CFA-1474290	Collagen formation	33 of 86	2.18e-09
CFA-216083	Integrin cell surface interactions	29 of 71	9.18e-09
CFA-3000171	Non-integrin membrane-ECM interactions	19 of 34	3.22e-07
CFA-3000178	ECM proteoglycans	21 of 44	3.38e-07
CFA-8948216	Collagen chain trimerization	20 of 41	5.21e-07
CFA-1474228	Degradation of the extracellular matrix	31 of 103	5.21e-07
CFA-1630316	Glycosaminoglycan metabolism	30 of 111	3.93e-06
CFA-381426	Regulation of Insulin-like IGF transport and uptake by IGF binding proteins	25 of 107	0.00030
CFA-186797	Signaling by PDGF	17 of 55	0.00042
CFA-201451	Signaling by BMP	9 of 25	0.0133
CFA-9006934	Signaling by Receptor Tyrosine Kinases	47 of 374	0.0374

Table 5. KEGG and Reactome pathway analysis of downregulated genes in ME cell lines in comparison to EP cell line.

KEGG Pathways – downregulated genes in mesenchymal-like cell lines			
Pathway	**Description**	**Count in gene set**	**FDR**
cfa04612	Antigen processing and presentation	13 of 51	0.00092
cfa04622	RIG-I-like receptor signaling pathway	13 of 52	0.00094
cfa04530	Tight junction	24 of 159	0.00094
cfa04514	Cell adhesion molecules (CAMs)	19 of 126	0.0041
cfa04670	Leukocyte transendothelial migration	16 of 106	0.0119
cfa00980	Metabolism of xenobiotics by cytochrome P450	10 of 47	0.0124
cfa05332	Graft-versus-host disease	7 of 24	0.0162
cfa05204	Chemical carcinogenesis	10 of 50	0.0165
cfa04064	NF-kappa B signaling pathway	13 of 84	0.0227
cfa04512	ECM-receptor interaction	11 of 76	0.0370
Reactome Pathways – downregulated genes in mesenchymal-like cell lines			
CFA-168256	Immune System	137 of 1477	3.28e-06
CFA-1169410	Antiviral mechanism by IFN-stimulated genes	16 of 39	4.72e-06
CFA-913531	Interferon Signaling	18 of 62	3.11e-05
CFA-1169408	ISG15 antiviral mechanism	14 of 35	3.11e-05
CFA-446728	Cell junction organization	16 of 60	0.00021
CFA-1500931	Cell-Cell communication	18 of 84	0.00045
CFA-9006934	Signaling by receptor tyrosine kinases	42 of 374	0.0025
CFA-1280218	Adaptive Immune System	60 of 625	0.0038
CFA-168249	Innate Immune System	74 of 836	0.0056
CFA-1280215	Cytokine Signaling in Immune System	39 of 369	0.0119
CFA-936440	Negative regulators of DDX58/IFIH1 signaling	7 of 18	0.0136

3.4 Discussion

In the present study, we characterized five canine mammary cancer cell lines regarding their morphology, intermediate filament expression and projections, invasiveness, tumorigenicity and gene expression of EMT-associated transcription factors. Using this model, we provide evidence that *ZEB1* and *ZEB2* are significantly associated with invasiveness and tumorigenicity in canine mammary cancer cells and a significant inverse correlation of *ZEB1* and *ZEB2* with *CDH1*, a marker of epithelial cells. Thus, we demonstrated that ME cancer cells are more malignant than the EP cells. Finally, upregulated differentially expressed genes in ME cell lines were functionally enriched to important pathways associated to EMT, invasiveness and tumorigenicity such as ECM organization and degradation, focal adhesion, TGF-Beta signaling, PI3K-AKT signaling, WNT signaling and regulation of insulin-like IGF transport and uptake by IGF binding proteins.

Zinc finger E-box Binding homeobox (ZEB) are transcription factors that present two zinc finger clusters that bind to specific DNA sequences and can either downregulate or upregulate the expression of specific genes (ZHANG; SUN; MA, 2015). In this study, we observed that higher expression of *ZEB1* and *ZEB2* in a metastatic canine mammary cancer cell line, CF41.Mg, is associated with higher tumorigenicity and invasiveness assessed by *in vitro* experiments. In accordance with our results, *ZEB1* and *ZEB2* is highly expressed in several human cancers such as breast, prostate, lung and oral cancers generally associated with invasion and metastasis by inducing EMT (VANDEWALLE et al., 2005; CHAFFER et al., 2013; CHU et al., 2013; LARSEN et al., 2016; SELTH et al., 2017). In addition, several evidences suggest that ZEB TFs, via genetic and epigenetic mechanisms, are associated with tumorigenicity, chemo- and radio-resistance and, consequently, tumor relapse in human cancer cells (POLYTARCHOUA; ILIOPOULOSA; STRUHLC, 2012; HASHIMOTO et al., 2016).

The role of ZEB1 in the generation and maintenance of tumorspheres was already demonstrated in human cancer. Two studies demonstrated the possible mechanisms by which ZEB1 could regulate the tumorigenicity in breast cancer. Chaffer *et al* determined that *ZEB1* promoter in non-tumorigenic breast cancer cells exhibit a bivalent chromatin configuration and could respond to signals such as TGF-β. This process triggers cell plasticity switching from a non-tumorigenic to a tumorigenic state (CHAFFER et al.,

2013). In the second study, it was shown that *ZEB1* promoted hypermethylation and silencing of *NGN3* gene, increasing the self-renewal potential of cancer cells (ZHOU et al., 2017). Our findings also support the hypothesis that *ZEB1* is a key factor for maintenance of tumorigenic potential in these cells. Here, only the ME cell lines (M5, M25 and CF41.Mg) which showed higher *ZEB1* expression, generated and maintained tumorspheres after 7 days of culture in low-adherent surfaces. Furthermore, CF41.Mg cells generate the biggest tumorspheres. These results suggest that *ZEB1* expression, as well as in human breast cancer is a key factor, at least *in vitro*, to tumorigenicity of CMC cells.

The correlation between *ZEB1* and *ZEB2* expression observed in our experiment, suggest that *ZEB2* also contributes to the tumorspheres formation in CMC cell lines. Despite presenting low expression in cells capable of forming tumorspheres such as M5 and M25, we observed higher *ZEB2* expression in CF41.Mg, the most malignant cell line in this experiment. These results support that co-expression of *ZEB1/2* transcription factors is important to maintain tumorigenicity properties, as stated by Chu and colleagues (CHU et al., 2013).

In human and murine cancer models, it was already demonstrated that *ZEB1* and *ZEB2* can downregulate epithelial markers such as E-cadherin and cytokeratin (AIGNER et al., 2007; LIU et al., 2008; SÁNCHEZ-TILLÓ et al., 2010; ROMERO-PÉREZ et al., 2013; WONG; GAO; CHAN, 2014). Our results support these studies as we found an inverse correlation among ZEB transcription factors and E-cadherin expression in the CMC cell lines. In addition, the ME cell lines (which expressed more *ZEB1/2*) expressed less or no cytokeratin when compared with EP cancer cells, supporting the role of *ZEB1/2* in the mesenchymal phenotype of canine mammary cancer cells.

We found only few studies relating *ZEB1/2* and canine mammary cancers. FR37-CMT cells isolated from a canine mammary tumor demonstrated a mesenchymal phenotype possibly regulated by a *TGF-β/ZEB1/miR-200* regulatory loop and exhibited resistance to cisplatin and doxorubicin (RAPOSO et al., 2017). Furthermore, FR37-CMT cells presented high invasion potential, corroborating with our results. Gamba and colleagues detected the expression of *ZEB1/2* in a spontaneous canine model of invasive micropapillary carcinoma (IMPC) of the mammary gland and showed that cytoplasmatic *ZEB2* predicted poor overall survival (GAMBA et al., 2014). On the other hand, they found *ZEB1* downregulation might be associated with the dedifferentiation process of

IMPC which is the opposite of what was found in human breast cancer (KARIHTALA et al., 2013). Thus, the present study contributed to determine the potential role of *ZEB1* and *ZEB2* as therapeutic targets in canine mammary cancers.

We also demonstrated that other pathways and genes allow greater invasiveness and tumorigenicity to ME cells. Upregulated differentially expressed genes in ME cells were enriched to pathways associated to ECM-receptor interaction, ECM organization and degradation, focal adhesion, TGF-β signaling, PI3K-Akt signaling, and WNT signaling. Genes associated with focal adhesion including *THBS2* and *ITGA8*, overexpressed in the ME cells, has been observed in another study of our group as potential genes mediating the invasion process in these cells (CORDEIRO et al., 2018). In particular, we also observed the overexpression of ECM degradation genes such as *MMP2*, *MMP14*, *MMP19*, *MMP24*, *ADAMTS4/5*, highly associated with metastasis and poor outcomes in different types of cancer (PORTER et al., 2004; MÜLLER et al., 2010; HILLEBRAND et al., 2019; NYANTE et al., 2019). Recently, was demonstrated that the deposition of collagen is very important to promote tumor growth and recurrence in breast cancer (WALENS et al., 2019), and, interestingly, a series of collagen family genes were upregulated in ME cells in our analysis.

Finally, TGF-β, BMP, WNT, and PI3K-Akt pathways are known to act like a tumor promoter stimulating EMT and contributing to cell invasion, tumorigenesis, and resistance to therapy (WU et al., 2012; XU; YANG; LU, 2015; COLAK; TEN DIJKE, 2017). Furthermore, these pathways are extremely linked to ZEBs expression and EMT process. TGF-β/BMP pathway induce ZEBs expression during EMT which interact to SMADs proteins to repress the expression of epithelial genes. Likewise, ZEB proteins play an important role in the regulation of TGF-β pathway during EMT (POSTIGO, 2003; POSTIGO et al., 2003). Interestingly, besides the high expression of ZEBs in ME cells, we also observed overexpression of *SMAD4/5/6/9*, and *BMP2* and *BMP4*. Furthermore, ZEB proteins can also be activated not only by TGF-β signaling but also by other pathways like WNT/β-catenin signaling (XU; LAMOUILLE; DERYNCK, 2009) that was also enriched in our analysis. Thus, our results corroborate and make a connection between EMT, invasiveness, and tumorigenicity in these canine mammary cancer cells.

3.5 Conclusion

In conclusion, the findings observed here support that *ZEB1* and *ZEB2* are possible key factors for invasiveness and tumorigenicity and potential candidates for further characterization in canine mammary cancers. This corroborates the studies in human breast cancers and highlights *ZEB*s transcription factors as targets for the development of new cancer therapies for humans and dogs. In addition, upregulated differentially expressed genes in ME cell lines were functionally enriched to pathways associated to EMT, invasiveness and tumorigenicity, justifying the higher malignance of these cells. Finally, our results suggested that the mechanisms responsible for obtaining these phenotypes are similar in canine and human mammary carcinomas, demonstrating a great potential of canine models for comparative and translational studies. Therefore, we have ongoing experiments in our laboratory to develop the best tool to silence gene expression of *ZEB* TFs in canine cancer cell lines that could be used in the future for clinical trials.

3.6 References

AIGNER, K. et al. The transcription factor ZEB1 (δEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. **Oncogene**, v. 26, n. 49, p. 6979–6988, 7 out. 2007. Disponível em: <http://www.nature.com/articles/1210508>.

COLAK, S.; TEN DIJKE, P. Targeting TGF-β Signaling in Cancer. **Trends in Cancer**, v. 3, n. 1, p. 56–71, 1 jan. 2017. Disponível em: <https://doi.org/10.1016/j.trecan.2016.11.008>.

CHAFFER, C. L. et al. XPoised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. **Cell**, v. 154, n. 1, p. 61–74, 2013. Disponível em: <http://dx.doi.org/10.1016/j.cell.2013.06.005>.

CHU, P. Y. et al. Epithelial-mesenchymal transition transcription factor ZEB1/ZEB2 co-expression predicts poor prognosis and maintains tumor-initiating properties in head and neck cancer. **Oral Oncology**, v. 49, n. 1, p. 34–41, 2013. Disponível em: <http://dx.doi.org/10.1016/j.oraloncology.2012.07.012>.

CORDEIRO, Y. G. et al. Transcriptomic profile reveals molecular events associated to focal adhesion and invasion in canine mammary gland tumour cell lines. **Veterinary and Comparative Oncology**, v. 16, n. 1, p. E89–E98, mar. 2018. Disponível em: <http://doi.wiley.com/10.1111/vco.12339>.

GAMBA, C. O. et al. ZEB2 and ZEB1 expression in a spontaneous canine model of invasive micropapillary carcinoma of the mammary gland. **Research in Veterinary Science**, v. 97, n. 3, p. 554–559, 2014. Disponível em: <http://dx.doi.org/10.1016/j.rvsc.2014.09.016>.

GAMBA, C. O. et al. The Relationship Between E-Cadherin and its Transcriptional Repressors in Spontaneously Arising Canine Invasive Micropapillary Mammary Carcinoma. **Journal of Comparative Pathology**, v. 153, n. 4, p. 256–265, 2015.

Disponível em: <http://dx.doi.org/10.1016/j.jcpa.2015.08.006>.

HASHIMOTO, A. et al. ZEB1 induces EPB41L5 in the cancer mesenchymal program that drives ARF6-based invasion, metastasis and drug resistance. **Oncogenesis**, v. 5, n. 9, p. e259, 2016. Disponível em: <http://dx.doi.org/10.1038/oncsis.2016.60>.

HELLMÉN, E. Complex mammary tumours in the female dog: A review. **Journal of Dairy Research**, v. 72, n. SPEC. ISS., p. 90–97, 2005.

HILLEBRAND, L. E. et al. MMP14 empowers tumor-initiating breast cancer cells under hypoxic nutrient-depleted conditions. **FASEB Journal**, v. 33, n. 3, p. 4124–4140, 2019.

KARIHTALA, P. et al. Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: Association with an aggressive tumour phenotype. **Breast Cancer Research and Treatment**, v. 138, n. 1, p. 81–90, 2013.

LARSEN, J. E. et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. **Journal of Clinical Investigation**, v. 126, n. 9, p. 3219–3235, 2016.

LIU, Y. et al. Zeb1 links epithelial-mesenchymal transition and cellular senescence. **Development**, v. 135, n. 3, p. 579–588, 2008.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. **Methods**, v. 25, n. 4, p. 402–408, 2001.

LUANPITPONG, S. et al. SLUG is required for SOX9 stabilization and functions to promote cancer stem cells and metastasis in human lung carcinoma. **Oncogene**, v. 35, n. 22, p. 2824–2833, 2016.

MÜLLER, M. et al. MMP19 is upregulated during melanoma progression and increases invasion of melanoma cells. **Modern Pathology**, v. 23, n. 4, p. 511–521, 2010.

NYANTE, S. J. et al. Quantitative expression of MMPs 2, 9, 14, and collagen IV in LCIS and paired normal breast tissue. **Scientific Reports**, v. 9, n. 1, p. 1–9, 2019. Disponível em: <http://dx.doi.org/10.1038/s41598-019-48602-6>.

PAOLONI, M.; KHANNA, C. Translation of new cancer treatments from pet dogs to humans. **Nature reviews. Cancer**, v. 8, n. 2, p. 147–56, 2008.

POLYTARCHOUA, C.; ILIOPOULOSA, D.; STRUHLC, K. An integrated transcriptional regulatory circuit that reinforces the breast cancer stem cell state. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 36, p. 14470–14475, 2012.

PORTER, S. et al. Dysregulated Expression of Adamalysin-Thrombospondin Genes in Human Breast Carcinoma. **Clinical Cancer Research**, v. 10, n. 7, p. 2429–2440, 2004.

POSTIGO, A. A. et al. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. **EMBO Journal**, v. 22, n. 10, p. 2453–2462, 2003.

QIAO, Y. et al. AP-1-mediated chromatin looping regulates ZEB2 transcription: New insights into TNFα-induced epithelial-mesenchymal transition in triple-negative breast cancer. **Oncotarget**, v. 6, n. 10, p. 7804–7814, 2015.

RAPOSO, L. R. et al. Immortalization and characterization of a new canine mammary tumour cell line FR37-CMT. **Veterinary and Comparative Oncology**, v. 15, n. 3, p.

952–967, 2017.

ROMERO-PÉREZ, L. et al. ZEB1 overexpression associated with E-cadherin and microRNA-200 downregulation is characteristic of undifferentiated endometrial carcinoma. **Modern Pathology**, v. 26, n. 11, p. 1514–1524, 2013.

SALAS, Y. et al. Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: A growing animal health problem. **PLoS ONE**, v. 10, n. 5, p. 1–15, 2015.

SÁNCHEZ-TILLÓ, E. et al. ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. **Oncogene**, v. 29, n. 24, p. 3490–3500, 2010.

SCHNEIDER, C. A.; RASBAND, W. S.; ELICEIRI, K. W. NIH Image to ImageJ: 25 years of image analysis. **Nature Methods**, v. 9, n. 7, p. 671–675, 28 jul. 2012. Disponível em: <http://www.nature.com/articles/nmeth.2089>.

SELTH, L. A. et al. A ZEB1-miR-375-YAP1 pathway regulates epithelial plasticity in prostate cancer. **Oncogene**, v. 36, n. 1, p. 24–34, 2017.

SLEECKX, N. et al. Canine mammary tumours, an Overview. **Reproduction in Domestic Animals**, v. 46, n. 6, p. 1112–1131, 2011.

SMITH, B. N. et al. Snail promotes epithelial mesenchymal transition in breast cancer cells in part via activation of nuclear ERK2. **PLoS ONE**, v. 9, n. 8, 2014.

TABASSUM, D. P.; POLYAK, K. Tumorigenesis: It takes a village. **Nature Reviews Cancer**, v. 15, n. 8, p. 473–483, 2015.

VALASTYAN, S.; WEINBERG, R. A. Tumor metastasis: Molecular insights and evolving paradigms. **Cell**, v. 147, n. 2, p. 275–292, 2011. Disponível em: <http://dx.doi.org/10.1016/j.cell.2011.09.024>.

VALLONE, P. M.; BUTLER, J. M. AutoDimer: A screening tool for primer-dimer and hairpin structures. **BioTechniques**, v. 37, n. 2, p. 226–231, 2004.

VANDEWALLE, C. et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. **Nucleic Acids Research**, v. 33, n. 20, p. 6566–6578, 2005.

WALENS, A. et al. CCL5 promotes breast cancer recurrence through macrophage recruitment in residual tumors. **eLife**, v. 8, p. 1–26, 2019.

WELLNER, U. et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. **Nature Cell Biology**, v. 11, n. 12, p. 1487–1495, 2009.

WONG, T. S.; GAO, W.; CHAN, J. Y. W. Transcription regulation of E-cadherin by zinc finger E-box binding homeobox proteins in solid tumors. **BioMed Research International**, v. 2014, 2014.

WU, Y. et al. Expression of Wnt3 activates Wnt/β-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. **Molecular Cancer Research**, v. 10, n. 12, p. 1597–1606, 2012.

XU, J.; LAMOUILLE, S.; DERYNCK, R. TGF-B-induced epithelial to mesenchymal transition. **Cell Research**, v. 19, n. 2, p. 156–172, 2009.

XU, W.; YANG, Z.; LU, N. A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. **Cell Adhesion and Migration**, v. 9, n. 4, p. 317–

324, 2015.

YE, J. et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. **BMC bioinformatics**, v. 13, n. May, p. 134, 2012.

ZHANG, P.; SUN, Y.; MA, L. ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. **Cell Cycle**, v. 14, n. 4, p. 481–487, 2015.

ZHOU, C. et al. ZEB1 confers stem cell-like properties in breast cancer by targeting neurogenin-3. **Oncotarget**, v. 8, n. 33, p. 54388–54401, 2017.

4. Chapter 3: An Epigenetic Screening Determines BET Proteins as Targets to Suppress Self-renewal and Tumorigenicity in Canine Mammary Cancer Cells

Pedro L. P. Xavier[1], Yonara G. Cordeiro[1], Pâmela A. Alexandre[1,2], Pedro R. L. Pires[1], Bruno H. Saranholi[3], Edson R. Silva[4], Susanne Müller[5], Heidge Fukumasu[1]

[1] Laboratory of Comparative and Translational Oncology (LOCT), Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, Brazil

[2] Present address: CSIRO Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation, Brisbane, Australia

[3] Department of Genetics and Evolution, Federal University of São Carlos, São Carlos, Brazil

[4] Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, Brazil

[5] Structural Genomics Consortium and Institute of Pharmaceutical Chemistry, Buchmann Institute for Molecular Life Sciences, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

Published in Scientific Reports 9, 17363. https://doi.org/10.1038/s41598-019-53915-7. 2019.

Abstract

Targeting self-renewal and tumorigenicity has been proposed as a potential strategy against cancer stem cells (CSCs). Epigenetic proteins are key modulators of gene expression and cancer development contributing to regulation and maintenance of self-renewal and tumorigenicity. Here, we have screened a small-molecule epigenetic inhibitor library using 3D *in vitro* models in order to determine potential epigenetic targets associated with self-renewal and tumorigenicity in Canine Mammary Cancer (CMC) cells. We identified inhibition of BET proteins as a promising strategy to inhibit CMC colonies and tumorspheres formation. Low concentrations of (+)-JQ1 were able to downregulate important genes associated to self-renewal pathways such as WNT, NOTCH, Hedgehog, PI3K/AKT/mTOR, EGF receptor and FGF receptor in CMC tumorspheres. In addition, we observed downregulation of *ZEB2*, a transcription factor important for the maintenance of self-renewal in canine mammary cancer cells. Furthermore, low concentrations of (+)-JQ1 were not cytotoxic in CMC cells cultured in

2D *in vitro* models but induced G2/M cell cycle arrest accompanied by upregulation of G2/M checkpoint-associated genes including *BTG2* and *CCNG2*. Our work indicates BET inhibition as a new strategy for canine mammary cancer by modulating the self-renewal phenotype in tumorigenic cells such as CSCs.

Keywords: BET proteins, canine mammary cancer, epigenetics, self-renewal, (+)-JQ1

4.1 Introduction

Mammary cancer in humans (HMC) and canines (CMC) share similar biological patterns, including high incidence, spontaneous development, associated risk factors, response to treatment and expression of molecular targets (PAOLONI; KHANNA, 2008; QUEIROGA et al., 2011). These features make dogs valuable models for comparative oncology and the development of new targets and therapies. Dogs have several advantages in comparison to other animal models such as generally sharing the same environment and being exposed to the same carcinogens as humans and thus influencing the epigenetic make-up (ROMAGNOLO et al., 2016). Therefore, canine cancer cell lines are useful models to study tumor behavior and development, characterize and validate novel molecular targets and aid at the development of potential anticancer molecules for HMC.

Apart from tumorigenicity, intra-tumor heterogeneity, the presence of different tumor cells, including cancer-stem cells (CSCs), in a single tumor, greatly influence tumor development (WAHL; SPIKE, 2017) . CSCs are rare cells within a tumor with the ability to self-renew, differentiate and tumor formation, underlying tumor initiation and progression (BATLLE; CLEVERS, 2017). Thus, considerable efforts to design innovative approaches to target these cells and their phenotypes have been made (BORAH et al., 2015). In order to enrich CSCs and, consequently, obtain a model to study and test approaches to target self-renewal and tumorigenicity, three-dimensional *in vitro* models (3D) using tumorspheres and colonies formation have been widely used (WEISWALD; BELLET; DANGLES-MARIE, 2015). However, in canine mammary cancer, few studies have addressed self-renewal and tumorigenicity phenotypes (COCOLA et al., 2009, 2017; RYBICKA; KRÏ¿½L, 2016). Recently, our group demonstrated that epithelial-mesenchymal transition (EMT)-associated transcription factors ZEB1 and ZEB2 are potential targets for the regulation of self-renewal and tumorigenicity of canine mammary cancer cells (XAVIER et al., 2018). However, to the

best of our knowledge, no chemical inhibitor for ZEB1/2 has thus far been developed (ZHANG et al., 2019).

Although cancer is typically considered a genetic disease, epigenetic abnormalities play an important role in the development and progression of cancer (JONES; BAYLIN, 2007). Thus, inhibitors targeting epigenetic modulators (typically referred to as writers, erasers and readers) have recently gained interest as potential and innovative therapeutic approaches in cancer therapy (MULLER; FILIPPAKOPOULOS; KNAPP, 2011; DAWSON; KOUZARIDES, 2012). In order to explore the therapeutic potential of novel epigenetic targets, specific inhibitors for a variety of epigenetic proteins have been developed. More than 50 specific inhibitors are available, covering particularly well the Bromodomain reader domains and epigenetic writers, histone lysine and arginine methyltransferases (SCHEER et al., 2019; WU et al., 2019).

The best-studied bromodomain family, is the bromodomain and extraterminal (BET) family of proteins. This family consists of four members: BRD2, BRD3, BRD4 and BRDT (FILIPPAKOPOULOS et al., 2010). Each of these proteins possesses two bromodomains that read acetyl-lysine residues and influence gene regulation, such as recruitment a complex of regulatory proteins, including positive transcription elongation factor b (P-TEFb) (YANG et al., 2005; MULLER; FILIPPAKOPOULOS; KNAPP, 2011; JOSLING et al., 2012). BET proteins have been shown to play key roles in human cancer and are considered attractive therapeutic targets. Several small molecules inhibitors of BET proteins, including (+)-JQ1 and iBETs, exhibit anti-neoplastic effects in cancers, such as acute myeloid leukemia (ZUBER et al., 2011), multiple myeloma (MERTZ et al., 2011), NUT midline carcinoma (ALEKSEYENKO et al., 2017), colon cancer (RODRIGUEZ et al., 2012) and breast cancer (OCAÑA; NIETO-JIMÉNEZ; PANDIELLA, 2017). BET proteins are also associated with hypoxia and tumor angiogenesis (DA MOTTA et al., 2017), epithelial-mesenchymal transition (EMT) (ANDRIEU; DENIS, 2018) and self-renewal (VENKATARAMAN et al., 2014). On the other hand, in companion animals no clinical study has been performed this far apart from a study using dogs as models to test the toxicity of the BET inhibitor CPI-0610 (ALBRECHT et al., 2016).

Here, we use an approach to evaluate epigenetic targets in canine mammary cancer cells and show that BET inhibition by (+)-JQ1 is a promising strategy to inhibit

self-renewal and tumorigenicity in CMC cells. Moreover, we demonstrate that BET proteins regulate the expression of genes associated with self-renewal and tumorigenicity pathways.

4.2 Material and Methods

4.2.1 Cell lines

Three cell lines were used in this experiment: M5 and M25 cells were isolated and characterized in our laboratory, as previously described (CORDEIRO et al., 2018), and the CF41.Mg cell line, kindly provided by Dr. Debora A. P. C. Zuccari (Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, São Paulo, Brazil). All CMC cells were maintained in 75 cm^2 flasks at 37ºC and 5% CO_2 with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Passaging was performed when cells were 85% confluent. Culture evolution was evaluated daily by optical microscopy (Axio Vert A1, Zeiss, Germany). All reagents used for cell culture were purchased from Thermo Fisher Scientific, USA.

4.2.2 Molecular Validation on CMC cells

In order to confirm the origin of cell lines, we amplified a fragment from cytochrome oxidase I (COI) and from 16S region using the primers described by Folmer (1994) and Palumbi et al. (1991) (FOLMER et al., 1994; PALUMBI et al., 2002). For both regions, the PCR reactions were performed at a final volume of 12 µL, containing: 0.2 mM of dNTPs, buffer tris-KCl 1X (Tris–HCl 20 mM pH 8.4 and KCl 50 mM), 2.5 mM of $MgCl_2$ (Invitrogen), 0.8 mM of each primer pair, 1 unit of Taq DNA Polymerase Platinum (Invitrogen), 50 ng of DNA template and ultrapure water (q.s.). The PCRs were conducted in Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation step at 94°C for 1 min; 35 cycles at 95°C for 30 s, the annealing temperature for each primer (50°C for COI and 57°C for 16S) for 1min and 72°C for 1 min; and a final extension step at 72°C for over 10 min. DNA from leukocytes of a known dog (*Canis lupus familiaris*) was used as positive control for PCRs. We also included a reference DNA from human and murine for the further cell lineage confirmation analysis. The amplification products were verified in electrophoresis on agarose gel at 1%. The PCR products were purified using ExoSAP-IT (Affymetrix)

and sequenced in an ABI3730XL sequencer (Applied Biosystems). The obtained sequences were analyzed and aligned with a reference sequence of a dog using the Geneious v.7.1.7 software (KEARSE et al., 2012). A sequence from *Felis catus* was used as outgroup (GenBank accession number FJ958339.1 – COI region, and GenBank accession number DQ983942 – 16S). The human and murine sequences obtained were also included as reference sequences. Genetic distances among the sequences were obtained using Kimura two-parameter model (KIMURA, 1980) and a neighbor-joining tree (SAITOU; NEI, 1987) was constructed using 1000 bootstrap replicates, both implemented in Geneious v.7.1.7 software (KEARSE et al., 2012) (**Supplementary Figure S1 and S2, Appendix C**). Also, the cell lineage confirmation was obtained comparing the obtained sequences with the sequences available in GenBank (BENSON et al., 2015) using the Blast tool (YE et al., 2012).

4.2.3 Epigenetic Probes Cytotoxic Assay

Epigenetic probes (Cayman Chemical, USA) (**Table 1**) were dissolved in DMSO to a concentration of 20 mM. The CF41.Mg cells were seeded at 2000/well in 96-well plates (Corning, USA) containing 100 µl of supplemented media as described. After 24h, media was replaced by new culture media containing different concentrations of epigenetic probes, ranging from 10 µM to 0.00064 µM. Epigenetic probes were added in six replicates per concentration. After 72h, 10 µl of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT - 5 mg/mL) was added to each well and formazan crystals were produced over a 2h incubation period. One hundred µl of DMSO were added to dissolve crystals. Optical density at 540 nm was measured in a Fluorstar Optima (BMG Labtech, Germany). The concentration of compounds resulting in IC_{50} was calculated using nonlinear regression test performed in GraphPad Prism (version 6.00 for Windows, GraphPad Software, USA).

4.2.4 Tumorspheres Formation Assay

Single cells were seeded into an ultra-low attachment surface 24-well plate (Corning) at a density of 8 x 10^2 cells suspended in 0.5 mL of serum-free DMEM-F12 supplemented with 1x B27 (Thermo Fisher Scientific), 20 ng/ml of EGF (PrepoTech, USA), 10 ng/ml of FGF (PrepoTech), 5 µg/ml of bovine insulin (Sigma Aldrich, USA), 4 µg/ml of heparin and 1% antibiotic/antimycotic. Tumorspheres number were evaluated

4 days after seeding. To generate secondary tumorspheres, primary tumorspheres were dissociated with trypsin (TrypLE Express Enzyme, Thermo Fisher Scientific). Single cells in suspension were seeded in the same density and evaluated 4 days after seeding. Pictures were taken with optical microscopy (Axio Vert A1, Zeiss).

4.2.5 Soft Agar Assay

Single cells were mixed in 0.3% agar (in DMEM-F12 supplemented with 10% FBS and 1% antibiotic/antimycotic) and plated at 1 x 10^4 onto 6-well plates containing a solidified bottom agar layer (0.6% agar in the same growth medium). Cells were maintained at 37ºC and 5% CO_2 for 14 days. Colonies were photographed in 10 pattern fields, counted, and measured using ZEISS ZEN 2 Microscope Software (ZEISS).

4.2.6 Real-Time PCR (qPCR)

Cells were treated with DMSO or 100 nM (+)-JQ1. After 72h, total RNA was extracted using Trizol® following the manufacturer's instructions. RNA samples were quantified and the 260/280 and 260/230 ratio (**Supplementary Table S1, Appendix C**) was assessed by NanoDrop 2000^{TM} (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription kit. Gene expression analyses were performed by real-time PCR using a StepOne System (Thermo Fisher Scientific). Specific primers were designed with Primer-BLAST (YE et al., 2012) and dimers and hairpins were verified using AutoDimer software (VALLONE; BUTLER, 2004). Primers were also analyzed by in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) to confirm specificity. Primer sequences are reported in **Supplementary table S2, Appendix C**. PCR reactions were carried out using Fast SYBR Green Master Mix in a final volume of 10 µl. Conditions for quantitative PCR were as follows: 95ºC for 20 s; 40 cycles at 95ºC for 3 s for denaturation, 60ºC for 30 s for anneal/extend; melt curve analysis was performed at 95ºC for 15 s and 60ºC for 60 s. The housekeeping gene used was the 18s ribosomal RNA and the analysis of relative gene expression data was performed according to the ΔΔCt method (LIVAK; SCHMITTGEN, 2001). Experiments were performed twice and in biological triplicates. All the reagents were purchased from Thermo Fisher Scientific.

4.2.7 In silico Analysis for Docking (+)-JQ1 into the Canine BET Proteins Structure

The amino acid sequences (FASTA) of human/canine BRD2 (NP_001106653.1 / NP_001041552.1), BRD3 (NP_031397.1 / XP_858014.1) and BRD4 (NP_490597.1 / XP_013977515.1) were compared by Protein Blast (ALTSCHUL et al., 1997). Computational analysis was performed using the crystal structure of the canine BET proteins co-crystallized with (+)-JQ1 (pdb 3MXF) (FILIPPAKOPOULOS et al., 2010). Receptor target and docking ligands were prepared using Chimera (PETTERSEN et al., 2004). The molecular surface of the target was generated based on the algorithm development (RICHARDS, 1977). Sphere generation was performed using the sphgen algorithm; the spheres were distributed with dock6 and selected using "spheres_selector". Grid generation was achieved using Grid, which is distributed as an accessory to DOCK (KUNTZ ID, BLANEY JM, OATLEY SJ, LANGRIDGE R, 1982). Flexible Dock was used to verify interactions between the target BET protein and (+)-JQ1 (MOUSTAKAS et al., 2006). Results obtained by docking were visualized and analyzed on Chimera version 1.4.1 (build 30365).

4.2.8 Cell Cycle Assay

The CF41.Mg cells were treated with DMSO (control) or 100 nM (+)-JQ1 for 72h. Cells were harvested and 1 x 10^6 cells were resuspended in cold PBS and fixed with absolute ethanol for 30 minutes. Cells were treated with 0.1% of Triton X-100 (Sigma Aldrich, USA), 20 µg/ml of propidium iodide (PI) (Thermo Fisher Scientific) and 200 µg/ml of RNase A (Thermo Fisher Scientific) for 30 minutes covered from light. Flow Cytometric Analysis was performed using S3e^{TM} Cell Sorter (Bio-Rad, USA). The data were analyzed using FCS Express 6 Flow Cytometry Software (De Novo Software, USA).

4.2.9 Cell Death Assay

To discriminate which type of cell death (+)-JQ1 induces in CF41.Mg cells (apoptosis versus oncosis) acridine orange assay was performed which is based on the arrangement of chromatin to differentiate apoptotic, oncotic and live cells. Live cells have normal nuclei staining which presents green chromatin with organized structures. Apoptotic cells contain condensed or fragmented chromatin (green or orange) and oncotic cells have similar normal nuclei staining as live cells except the chromatin is orange instead of green (RIBBLE et al., 2005). The CF41. Mg cells were seeded in 6-well plates

and after 24 h, cells were treated with DMSO or (+)-JQ1 at a final concentration of 4 µM, 1 µM, 300 nM, 150 nM and 100 nM for 72h. A dye mix containing 100 µg/ml of acridine orange and 100 µg/ml of ethidium bromide was added to cells and observed for fluorescence emission using ZEISS—Axio Vert A1 with a camera Axio Can 503 attached using a 520 nm and 620 nm wavelength filter for green and red colors, respectively (ZEISS). Analyses were performed in triplicate, counting a minimum of 100 total cells each.

4.2.10 RNA-seq Data Generation

Tumorspheres treated with 100 nM (+)-JQ1 or 100 nM (-)-JQ1 were collected after 4 days of culture and the RNA was extracted using RNeasy Mini Kit (QIAGEN, UK). The RNA quality and quantity were assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to the manufacturer's instructions (Agilent Technologies, Ireland). Only samples that presented an RNA integrity number (RIN) higher than 8.0 were considered to the sequencing (**Supplementary Table S3, Appendix C**). RNA libraries were constructed using the TruSeq™ Stranded mRNA LT Sample Prep Protocol and sequenced on Illumina HiSeq 2500 equipment in a HiSeq Flow Cell v4 using HiSeq SBS Kit v4 (2x100pb).

4.2.11 Alignment and Differential Expression

Sequencing quality was evaluated using the software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and no additional filter was performed. Sequence alignment against the canine reference genome (CanFam3.1) was performed using STAR (DOBIN et al., 2013), according to the standard parameters and including the annotation file (Ensembl release 89). Secondary alignments, duplicated reads and reads failing vendor quality checks were removed using Samtools (LI et al., 2009). Alignment quality was confirmed using Qualimap (GARCÍA-ALCALDE et al., 2012). Gene expression was estimated by read counts using HTseq (ANDERS; PYL; HUBER, 2015) and normalized as counts per million reads (CPM). Only genes presenting at least 1 CPM in at least 6 samples were kept for differential expression (DE) analysis. DE was performed using edgeR package (ROBINSON; MCCARTHY; SMYTH, 2009) on R environment, based on negative binomial distribution. Benjamini-Hochberg procedure was used to control the false discovery rate (FDR) and transcripts presenting FDR $\leq$ 0.01 and log-fold change (LogFC) >1;<-1 were considered differential expressed

(DE). Functional enrichment analysis of DE genes was performed using STRING (JENSEN et al., 2009; SZKLARCZYK et al., 2019).

4.2.12 Statistical Analysis

The IC_{50} was calculated using nonlinear regression test. Gene expression, colonies and tumorsphere formation were analyzed by one-way ANOVA with post hoc Tukey. Unpaired T-test was used for gene expression analysis of non-treated and (+)-JQ1-treated cells. For functional enrichment analyses, *p*-value was adjusted for multiple tests, and Benjamini and Hochberg method was used to test multiple categories in a group of functional gene sets. Significant differences were considered when $p < 0.05$.

4.3 Results

4.3.1 Effect of epigenetic inhibitors on CMC cells

An initial screening was performed in order to determine the cytotoxic potential of a small library of 27 epigenetic inhibitors in the CF41.Mg cell line, considered the most malignant canine mammary cancer cell line of our cell bank, with higher tumorigenicity and self-renewal potential compared to the other cell lines (XAVIER et al., 2018). From the 27 epigenetic inhibitors tested, only (+)-JQ1, NVS-CECR2-1 and UNC1999 showed an IC_{50} lower than 10 μM **(Table 1)**. According to the results, we set the non-cytotoxic concentration of 1μM for all probes for the next experiments, which aim to observe the potential of the epigenetic inhibitors regarding tumorigenicity and self-renewal using 3D *in vitro* models.

Table 1. List of 27 epigenetic inhibitors, their targets and IC_{50} values.

Inhibitor	Specific targets	Target Enzymatic Class	IC_{50} (μM)
(+)-JQ1	BRD2, BRD3, BRD4, BRDT(BET)	Bromodomains	3.9
GSK2801	BAZ2B/A	Bromodomains	>10
NI-57	BRPF	Bromodomains	>10
PFI-3	SMARCA2/4 e PB1/5	Bromodomains	>10
BAY-598	SET e SMYD2	Methyltransferases	>10
GSK-J4	JMJD3	Demethylases	>10
NVS-CECR2-1	CECR2-1	Bromodomains	3.98
GSK 484	PAD4	Deiminases	>10
SGC0946	DOT1L	Methyltransferases	>10
OICR-9429	WDR5	WD40	>10
R-PFI-2	SETD7	Methyltransferases	>10
MS049	PRMT4/6	Methyltransferases	>10
GSK864	IDH1	IDH1 mutant inhibitor	>10
GSK343	EZH2	Methyltransferases	>10
GSK-LSD1	LSD1	Demethylases	>10
MS023	PRMTs	Methyltransferases	>10
UNC1215	L3MBTL3	Methylated Lysines reader	>10
Bi-9564	BRD9/7	Bromodomains	>10
BAZ2-ICR	BAZ2A/B	Bromodomains	>10
UNC-1999	EZH2/1	Methyltransferases	4.70
TP-064	PRMT4	Methyltransferases	>10
A-196	SUV420H1/H2	Methyltransferases	>10
A-366	G9a/GLP	Methyltransferases	>10
PFI-4	BRPF1B	Bromodomains	>10
SGC-CBP30	CREBBP e EP300	Acetylases	>10
SGC-707	PRMT3	Methyltransferases	>10
GSK591	PRMT5	Methyltransferases	>10

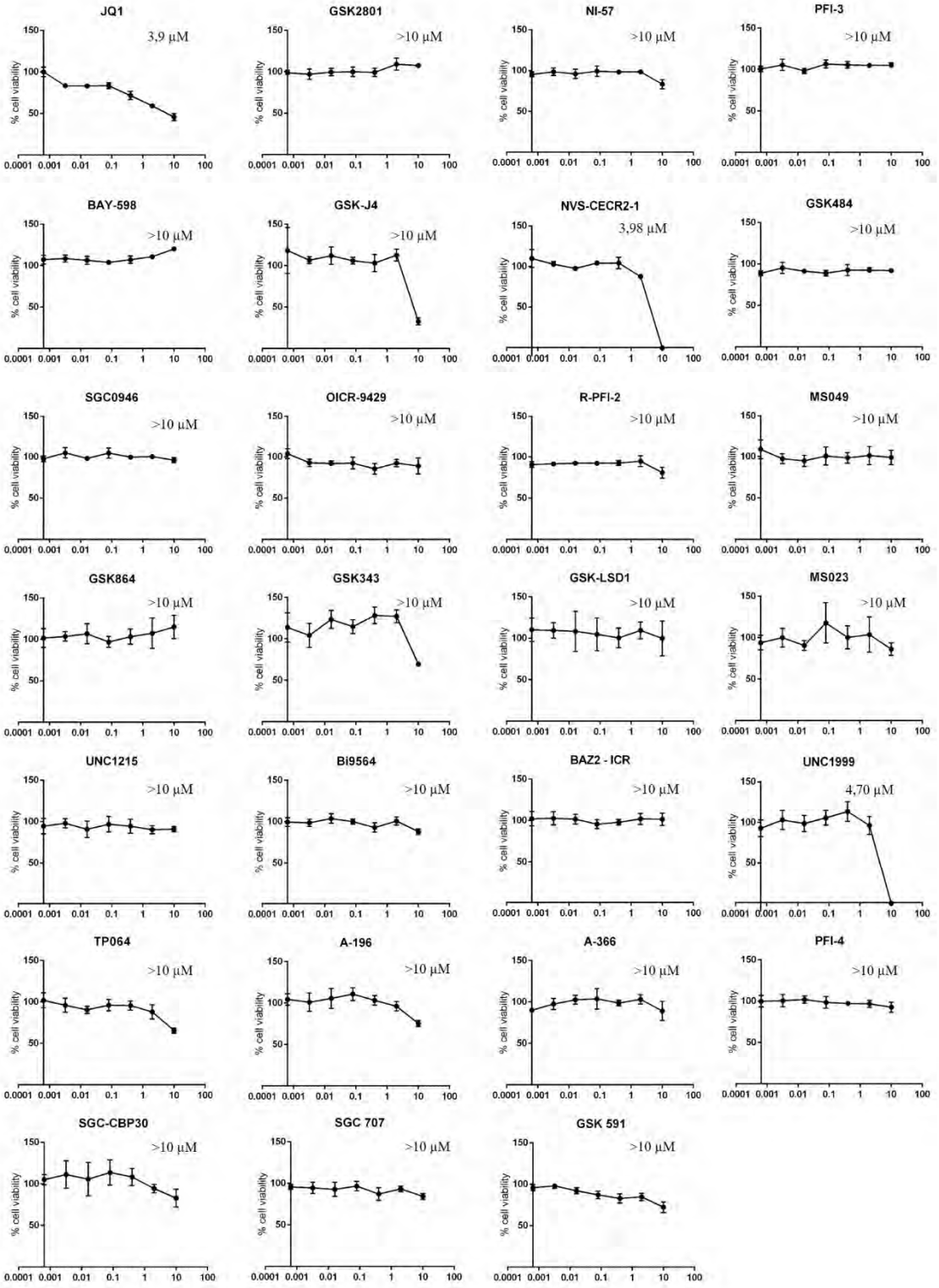


Figure 1. IC_{50} of 27 epigenetic inhibitors in CF41.Mg cells. Only the (+)-JQ1, GSK-J4, NVS-CECR2-1, and UNC1999 probes showed cytotoxic potential in concentrations lower or equal to 10 µM.

4.3.2 Assessment of epigenetic inhibitors on 3D *in vitro* models

Next, we aimed to explore the effects of epigenetic inhibitors regarding tumorigenicity and self-renewal of CF41.Mg cells using the tumor-cell colony formation in soft agar assay and the tumorsphere formation assay. From the 27 epigenetic inhibitors tested at 1 μM only (+)-JQ1, NVS-CECR2-1, GSK343, UNC1999 and A-196 decreased the number of colonies in soft agar when compared to the control treatment (**Figure 2A,** $p < 0.05$) (**Supplementary Figure S3, Appendix C**). However, only (+)-JQ1 was effective in reducing both the number and size of colonies in soft agar (**Figure 2B**, $p < 0.05$). Therefore, these 5 epigenetic inhibitors were used in the assay for formation of primary and secondary tumorspheres, in which only (+)-JQ1 and NVS-CECR2-1 (at 1 μM) showed a significant inhibitory effect to primary tumorsphere formation (**Figure. 2C**; $p < 0.05$) (**Supplementary figure S4, Appendix C**). Both (+)-JQ1 and NVS-CECR2-1 nearly totally inhibited primary tumorspheres formation, while GSK343, UNC1999 and A-196 showed no inhibitory effect for primary and secondary tumorsphere formation **(Figure 2C and 2D**) (**Supplementary figure S5, Appendix C**). Thus, (+)-JQ1 and NVS-CECR2-1 showed the most potent inhibitory effects in two 3D experiments and were selected for further investigation.

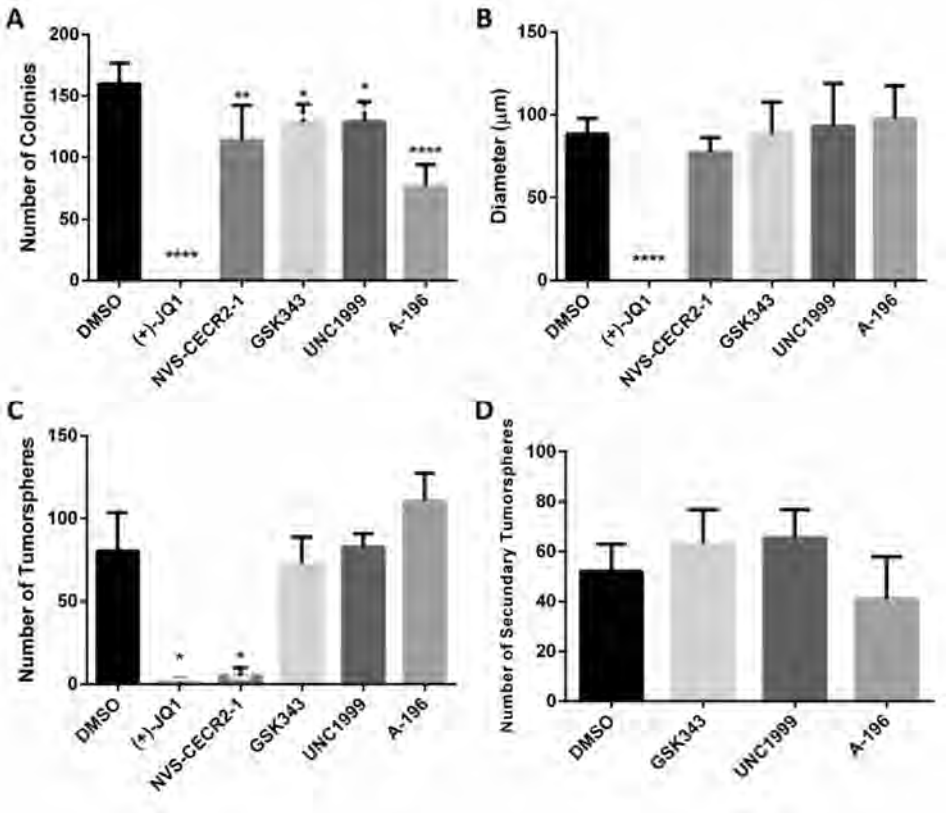


Figure 2. Number and size of colonies and number of primary and secondary tumorspheres. (A) (+)-JQ1, NVS-CECR2-1, GSK343, UNC1999 and A-196 decreased the number of colonies in comparison to the control (DMSO) formed in soft agar assay. (**B)** Only (+)-JQ1 decreased the diameter of colonies in comparison to the control (DMSO). Only ≥50 μm colonies were counted. (**C)** (+)-JQ1 and NVS-CECR2-1 inhibit tumorsphere formation in low-adherent plates. No difference was observed to tumorspheres treated with GSK343, UNC1999, and A-196 in comparison to the control (DMSO). (**D)** GSK343, UNC1999 and A-196 were also unable to inhibit the formation of secondary tumorspheres. (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; - One-way ANOVA followed by Tukey's multiple comparison test).

We then evaluated the minimal concentration necessary to fully inhibit anchorage-independent cell growth. We tested (+)-JQ1 and NVS-CECR2-1 at lower concentrations in a dose dependent manner. (+)-JQ1 was able to fully inhibit the growth of colonies at the concentration of 300 nM, whereas at concentrations of 150 nM or 100 nM the number and size of the colonies was merely decreased (**Figure 3A-B**; $p < 0.0001$). All (+)-JQ1 concentrations also inhibited the formation of tumorspheres (**Figure 3C-D**; $p < 0.0001$) and concentrations of 150 nM or 100 nM of (+)-JQ1 reduced the number of secondary tumorspheres in comparison with the control (**Figure 3D**; $p < 0.0005$). In order to confirm the specificity of the results, we next tested the inactive stereoisomer of (+)-JQ1, (-)-JQ1, at the same concentrations in CF41.Mg cells. This molecule has virtually the same physical and chemical structures as (+)-JQ1 but is unable to inhibit BET family bromodomains. Accordingly, there was no difference in the number of CF41.Mg tumorspheres between control and cells treated with (-)-JQ1, confirming that the effect on tumorsphere growth inhibition is due to inhibition of BET proteins (**Figure 3E**). Surprisingly, lower doses of NVS-CECR2-1 did not have the same effect on the growth of CF41.Mg tumorspheres (**Supplementary figure S6, Appendix**). Upon closer inspection, we observed precipitates at concentrations above 1 μM, which can lead to cell death, justifying the initial result observed. Thus, we decided to concentrate of (+)-JQ1 in the further experiments.

Several reports show that at high concentrations (+)-JQ1 induces apoptosis and cell cycle arrest in human cancer cells (FILIPPAKOPOULOS et al., 2010; LI et al., 2018; XU et al., 2018). Also, in CF41.Mg canine cells, concentrations of 1 μM and above of (+)-JQ1 induced apoptosis, whereas lower concentrations of 300 nM and below have no apoptotic effects (**Figure 3F; Supplementary figure S7A, Appendix C**; $p < 0.05$). To explore the mechanism by which (+)-JQ1 inhibits colony and tumorspheres formation,

we performed cell cycle analysis using flow cytometry. The cell cycle of CF41.Mg cells was analyzed using (+)-JQ1 at concentrations not inducting apoptosis. We established that (+)-JQ1 treatment induced a G2/M cell cycle arrest in these cells (**Figure 3G**; **Supplementary figure S7B, Appendix C;** $p < 0.05$), suggesting a possible mechanism for the inhibition of the CF41.Mg tumorspheres and colonies.

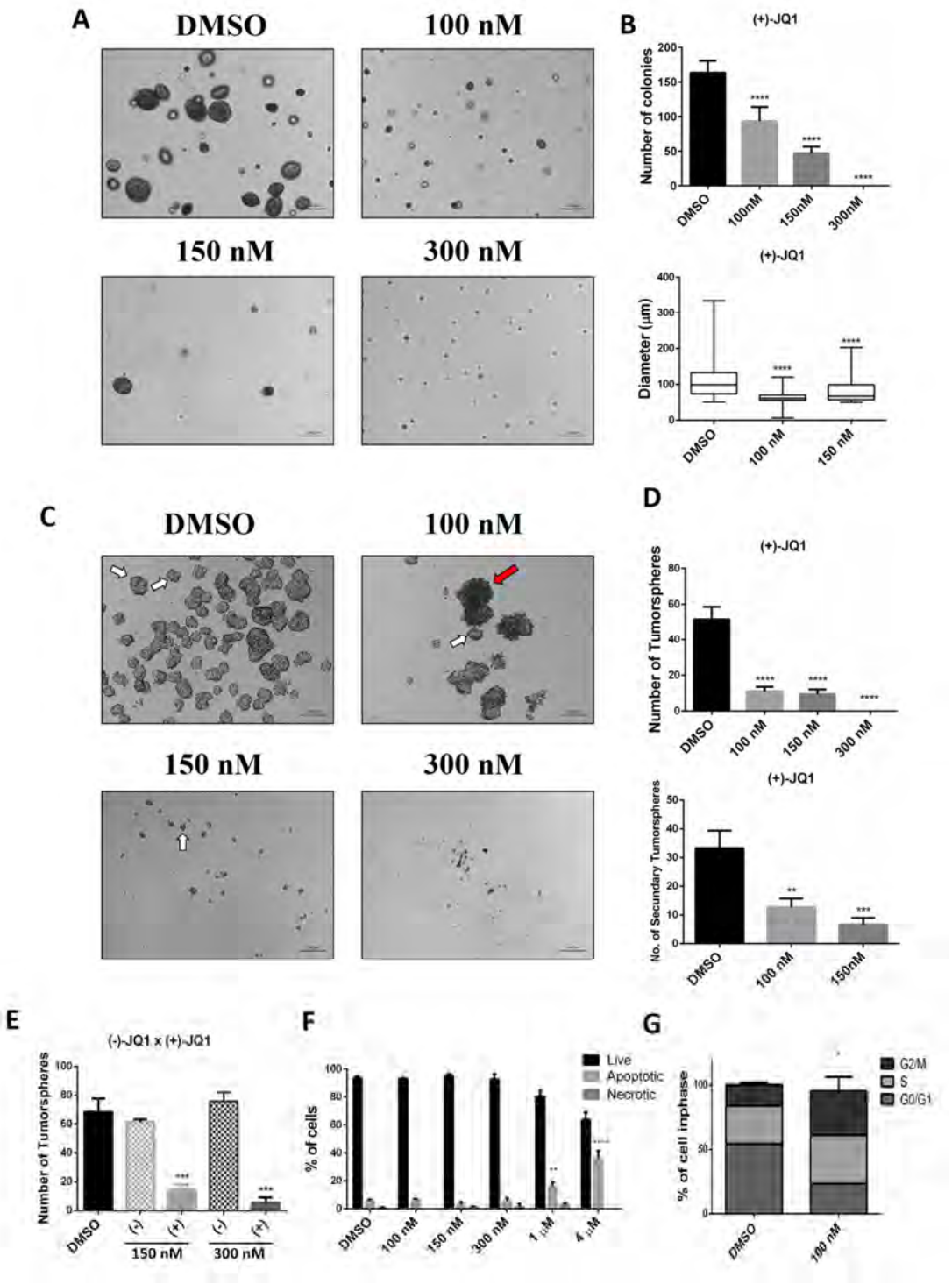


Figure 3. Effects of (+)-JQ1 regarding colonies formation, tumorsphere formation, cell death and cell cycle. (A-B) (+)-JQ1 used at concentrations of 100 nM, 150 nM and 300 nM were able to decrease the number and size of colonies in comparison to control. Only ≥50 μm colonies were counted. (**C-D)** In addition, (+)-JQ1 was able to inhibit the number of primary and secondary tumorspheres in comparison to the control. White arrows represent tumorspheres while red arrow represent cell aggregates. (**E)** The stereoisomer of (+)-JQ1, (-)-JQ1, at concentrations of 150 nM and 300 nM did not inhibit the tumorspheres formation. (**F**) One μM or 4 μM, respectively of (+)-JQ1 induced the increasing of apoptotic CF41.Mg cells. On the other hand, 300 nM, 150 nM and 100 nM of (+)-JQ1 showed no difference in comparison to the control. (**G)** After 72h of (+)-JQ1 treatment, flow cytometry analyses for CF41.Mg cells show increase G2/M cell cycle arrest in (+)-JQ1 treated cells compared to the control. (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ – One-way ANOVA followed by Tukey's multiple comparison test).

We next confirmed the effect of (+)-JQ1 on tumorspheres in two other canine mammary cancer cell lines with tumorsphere potential, M5 and M25. (+)-JQ1 reduced the number of primary and secondary tumorspheres in M5 cells at both concentrations tested (150 nM and 300 nM) (**Supplementary figure S8, Appendix C**). In M25 cells, the number of secondary tumorspheres was reduced when treated with doses of 300 nM (+)-JQ1 (**Supplementary figure S9, Appendix C**).

4.3.3 Transcriptomic analysis of (+)-JQ1-treated tumorspheres

In order to assess the genes affected by treatment with (+)-JQ1 in tumorspheres, we treated CF41.Mg tumorspheres with 100 nM of the inhibitor. An average of 19.8 million paired-end reads were sequenced per replicate (3 replicates per tumorspheres condition) and an average of 90% were aligned to the reference genome as concordant pairs (**Supplementary Table S4, Appendix C**). A total of 11.620 genes passed quality control and were tested for differential expression (DE). Of these 516 genes were downregulated and 444 were upregulated in (+)-JQ1-treated tumorspheres (FDR< 0.01 and LogFC > 1;<-1), demonstrating the impact of (+)-JQ1 in gene expression modulation on CF41.Mg tumorspheres even at a low dose (**Figure 4A**). The top 25 up- and downregulated genes are exhibited in **Table 2**. Interestingly, we found some of the top downregulated genes by (+)-JQ1 associated with self-renewal including Thrombospondin-2 (*THBS2*) (LogFC = -6.01), *ETV7* (LogFC = -4.31), Dickkopf-related protein 1 (*DKK1*) (LogFC = -4.12) and ROS proto-oncogene 1 (*ROS1*) (LogFC = -4.07) (AHN et al., 2008; SINGER et al., 2015; KUO et al., 2018; NUMATA; KLEIN GELTINK; GROSVELD, 2019). Functional enrichment analysis showed that DE genes between tumorspheres treated with (+)-JQ1

and (-)-JQ1 were related to KEGG and Reactome pathways such as proteoglycans in cancer, pathways in cancer, microRNAs in cancer, extracellular matrix organization, degradation of the extracellular matrix and regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs). A full list of enriched terms is reported in **Table 3**.

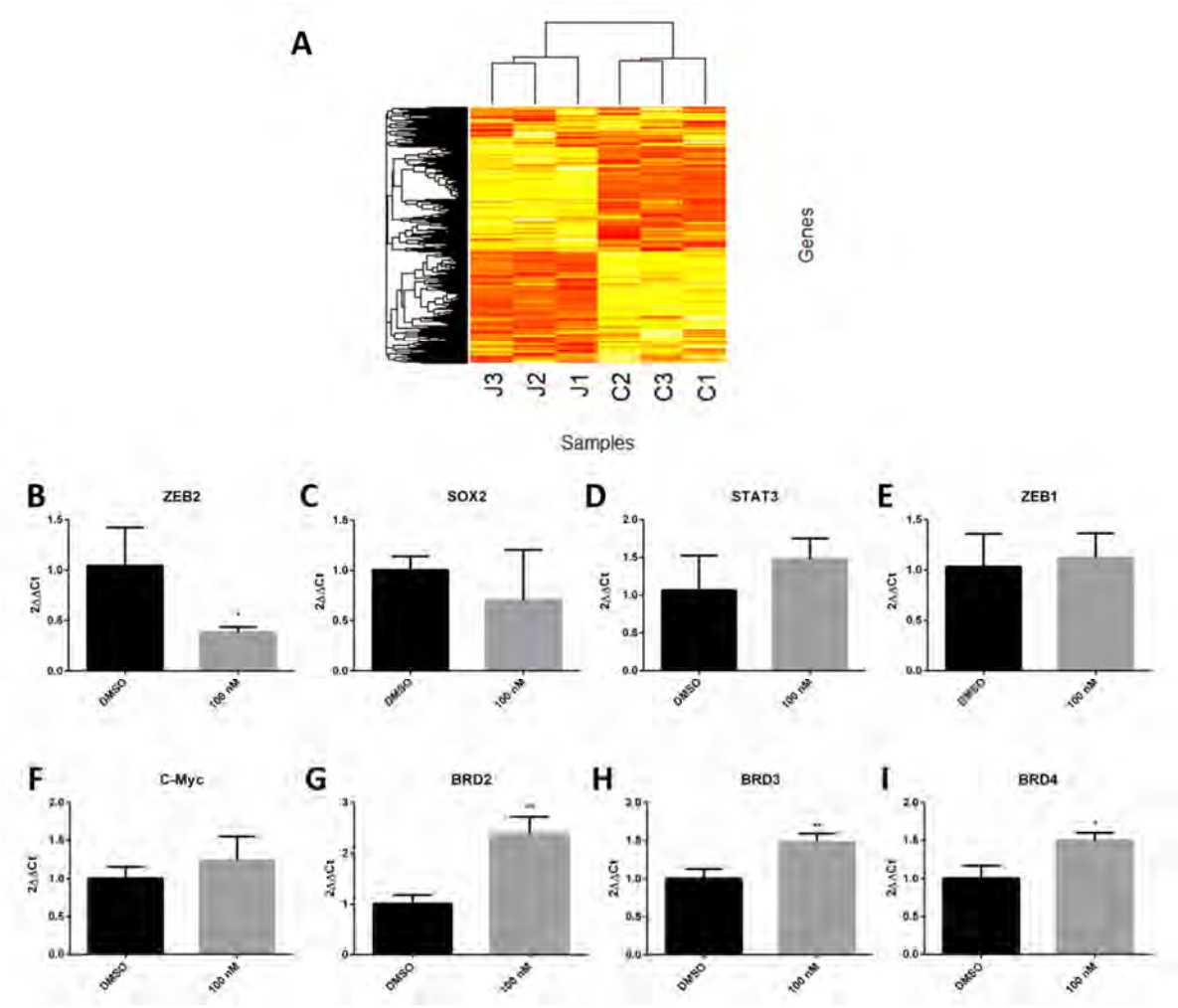


Figure 4. Gene expression analysis in (+)-JQ1-treated and non-treated CF41.Mg tumorspheres and adhrente cells. (**A**) Heatmap of all differentially expressed (DE) genes between (+)-JQ1-treated tumorspheres and (-)-JQ1-treated tumorspheres demonstrating the impact of (+)-JQ1 on modulation of gene expression in CF41.Mg tumorspheres. (**B**) (+)-JQ1 treatment significantly decreased *ZEB2* expression. (**C-E**) (+)-JQ1 treatment had no effect on gene expression of key stem cell associated genes, *SOX2*, *STAT3*, *ZEB1 and c-Myc*. (**F-I**) (+)-JQ1 treatment significantly increased the levels of *BRD2*, *BRD3* and *BRD4* gene expression. The *18S* gene was used as the housekeeping gene. (*$p < 0.05$; ** $p < 0.01$; - Unpaired T test).

Table 2. The top 25 down and upregulated genes in 100 nM (+)-JQ1-treated tumorspheres in comparison with control tumorspheres.

Ensembl ID	Gene name	logFC	FDR
ENSCAFG00000007251	*KRT5*	-7.18	1.81E-78
ENSCAFG00000015625	*FMN2*	-6.44	~ 0
ENSCAFG00000000874	*THBS2*	-6.01	3.46E-94
ENSCAFG00000018405	*FST*	-5.98	~ 0
ENSCAFG00000040526		-5.90	7.91E-61
ENSCAFG00000008359	*DCHS2*	-5.49	3.50E-154
ENSCAFG00000031666	*CLSTN3*	-5.43	1.92E-56
ENSCAFG00000006142	*DCN*	-5.30	~ 0
ENSCAFG00000024288	*TRABD2B*	-5.30	1.15E-107
ENSCAFG00000033166		-5.20	3.64E-33
ENSCAFG00000005068	*KCTD12*	-5.03	3.34E-21
ENSCAFG00000003684	*UCP1*	-5.01	3.97E-106
ENSCAFG00000006674	*SPINK5*	-5.00	1.68E-22
ENSCAFG00000017871	*FAT2*	-4.38	4.05E-39
ENSCAFG00000009666	*ZPLD1*	-4.38	6.80E-40
ENSCAFG00000005012	*TMEM163*	-4.38	6.24E-39
ENSCAFG00000001394	*ETV7*	-4.32	5.70E-20
ENSCAFG00000008335		-4.30	1.05E-24
ENSCAFG00000031918	*TMEM26*	-4.22	3.76E-40
ENSCAFG00000015553	*DKK1*	-4.12	6.80E-40
ENSCAFG00000000923	*ROS1*	-4.08	1.50E-52
ENSCAFG00000032756	*FAM198b*	-3.96	1.02E-127
ENSCAFG00000017137	*TENM2*	-3.95	4.58E-51
ENSCAFG00000006138	*LUM*	-3.93	~ 0
ENSCAFG00000015708	*ACTA2*	-3.93	2.33E-21
ENSCAFG00000009258	*CYTIP*	5.54	4.34E-21
ENSCAFG00000007018	*DEPP1*	5.27	3.18E-37
ENSCAFG00000014752	*TFP1*	4.64	1.88E-25
ENSCAFG00000013306	*APO4*	4.60	7.08E-24

ENSCAFG00000006413	*NCKAP1L*	4.31	1.39E-30
ENSCAFG00000001091	*TGFB1*	4.11	2.46E-49
ENSCAFG00000001106	*LAMA2*	4.10	7.64E-20
ENSCAFG00000009333	*CES1*	3.65	1.71E-77
ENSCAFG00000018146	*ALDH3A1*	3.54	4.63E-137
ENSCAFG00000017937	*CYP1A1*	3.39	7.32E-76
ENSCAFG00000018218	*AMOT*	3.30	1.25E-14
ENSCAFG00000009421	*MMP2*	3.19	2.76E-98
ENSCAFG00000001007	*FBXO32*	3.17	5.15E-119
ENSCAFG00000029721	*ZNF132*	3.15	8.90E-18
ENSCAFG00000029558	*BMF*	3.02	2.99E-86
ENSCAFG00000030120	*CSPG4*	2.96	1.57E-154
ENSCAFG00000010888	*UPK1B*	2.92	2.83E-70
ENSCAFG00000012396	*PHYHIPL*	2.91	7.23E-19
ENSCAFG00000031014	*FAM180A*	2.85	1.65E-18
ENSCAFG00000018047		2.82	6.75E-33
ENSCAFG00000011236	*AXIN2*	2.77	9.49E-32
ENSCAFG00000011002	*TINAGL1*	2.76	2.50E-256
ENSCAFG00000011595	*ARHGAP6*	2.75	2.07E-21
ENSCAFG00000006977	*ABTB2*	2.70	3.92E-82
ENSCAFG00000011913	*LAMB3*	2.64	5.13E-13

Table 3. KEGG and Reactome pathway analysis of DE genes between 100 nM (+)-JQ1-treated tumorspheres and control tumorspheres.

KEGG and Reactome pathways			
Pathway	**Description**	**Count in gene set**	**FDR**
cfa05418	Fluid shear stress and atherosclerosis	19 of 125	0.0022
cfa05205	Proteoglycans in cancer	24 of 183	0.0022
cfa05200	Pathways in cancer	45 of 475	0.0022
cfa05206	MicroRNAs in cancer	18 of 134	0.0070
cfa05146	Amoebiasis	13 of 86	0.0182
cfa04512	ECM-receptor interaction	12 of 76	0.0182
cfa04360	Axon Guidance	19 of 164	0.0182
cfa04933	AGE-RAGE signaling pathway	13 of 93	0.0221
cfa00340	Histidine Metabolism	6 of 22	0.0323
cfa04750	Inflammatory mediator regulation of TRP Channels	12 of 89	0.0373
cfa1474244	Extracellular matrix organization	31 of 253	0.0019
cfa422475	Axon guidance	30 of 275	0.0106
cfa381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	15 of 107	0.0334
cfa2022090	Assembly of collagen fibrils and other multimeric structures	11 of 58	0.0334
cfa1630316	Glycosaminoglycan metabolism	16 of 111	0.0334
cfa162582	Signal Transduction	135 of 2177	0.0334
cfa1474290	Collagen formation	13 of 86	0.0334
cfa1442490	Collagen degradation	10 of 46	0.0334
cfa1266738	Developmental Biology	37 of 415	0.0334
cfa8874081	MET activates PTK2 signaling	7 of 26	0.0373
cfa8875878	MET promotes cell motility	8 of 36	0.0405

In our analysis, we also identified genes associated with G2/M cell cycle checkpoint and self-renewal when comparing the data set with information from publicly available data from the Molecular Signatures DataBase (MSigDB,

http://www.broadinstitute.org/gsea/msigdb) and PANTHER classification system. We found important G2/M checkpoint-associated genes upregulated by (+)-JQ1 including B-cell Translocation Gene 2 (*BTG2*), Cyclin-G2 (*CCNG2*) and Epidermal Growth Factor Receptor (*EGFR*). (+)-JQ1 also increased the gene expression of Bone Morphogenetic Protein 7 (*BMP7*) and BCL-2-like protein 11 (*BCL2L11*), commonly called Bim, both associated with programmed cell death. Finally, we observed that (+)-JQ1 downregulated genes of important self-renewal pathways including WNT, NOTCH, Hedgehog, PI3K/AKT/mTOR, EGF receptor and FGF receptor, such as *GNB4*, *TLE2*, *BMPR1B*, *CDH10*, *ACVR1B*, *ACTA2*, *FAT2*, *WNT9A*, *CDH13*, *PRKD1*, *PIK3CD*, *ERBB3*, *NRG1*, *GNB4*, *GNB5*, *NOS3*, *EIF4E3*, *PTPN6*, *PTCH1*, *BMP4* and *NOTCH1* (**Table 4**). Furthermore, we observed downregulation of *ZEB2*, a transcription factor important for the maintenance of self-renewal in canine mammary cancer cells expression (XAVIER et al., 2018).The decrease of *ZEB2* expression in (+)-JQ1-treated CF41.Mg cells was also observed by qPCR analysis (**Figure 4B**; $p < 0.05$). However, the expression levels of some self-renewal associated genes such as *SOX2*, *STAT3*, *ZEB1* and *c-Myc*, a key target gene of BET proteins, showed no significant difference after (+)-JQ1 treatment (**Figure 4C-F**). Interestingly, (+)-JQ1 treatment significantly increased gene expression of *BRD2*, *BRD3* and *BRD4* in CF41.Mg cells (**Figure 4G-I**; $p < 0.05$), which may present a compensatory response after BET proteins inhibition by the probe.

These results demonstrate that BET inhibition by (+)-JQ1 can modulate key genes associated with self-renewal and G2/M checkpoint in CMC cells corroborating the decrease of tumorspheres and colonies accessed by 3D *in vitro* models.

Table 4. Self-renewal-associated genes downregulated by (+)-JQ1.

Self-renewal-associated genes			
Pathway	**Gene**	**LogFC**	**FDR**
WNT	*GNB4*	-1.16	2.24E-30
	TLE2	-1.85	4.98E-24
	BMPR1B	-1.12	4.27E-11
	CDH10	-2.96	6.20E-15
	ACVR1B	-1.06	2.07E-18
	ACTA2	-3.93	2.33E-21
	FAT2	-4.38	4.05E-39
	WNT9A	-1.51	1.43E-21
	CDH13	-3.85	9.99E-19
EGFR	*PRKD1*	-2.16	6.72E-11
	PIK3CD	-1.49	5.05E-60
	ERBB3	-2.28	9.11E-15
	NRG1	-1.05	1.85E-67
PI3K/AKT/mTOR	*GNB4*	-1.16	2.24E-30
	GNB5	-1.08	5.11E-14
	NOS3	-2.59	2.73E-16
	EIF4E3	-2.38	7.08E-09
FGFR	*PTPN6*	-2.06	1.08E-26
	PIK3CD	-1.49	5.05E-60
Hedgehog	*PTCH1*	-1.07	4.86E-26
	BMP4	-1.28	1.03E-84
NOTCH	*NOTCH1*	-1.00	8.03E-77

4.3.4 Canine BET proteins: gene expression and homology

BET proteins are extremely conserved between species and also their expression patterns has been found to be conserved (TANIGUCHI, 2016). In order to confirm the expression of *BRD2*, *BRD3* and *BRD4* in CMC cells we performed qPCR analysis. *BRD2*, *BRD3* and *BRD4* genes were expressed in CMC cells with *BRD2* being the most expressed gene (**Figure 5A**). All three cell lines, M5, M25 and CF41.Mg, showed high

expression of *BRD2*, *BRD3* and *BRD4* (**Table 5**) with no difference in expression level between the cell lines (**Figure 5B**).

The inhibitor (+)-JQ1 was designed based on the acetylated lysine binding sites of human BET proteins (FILIPPAKOPOULOS et al., 2010). Thus, we performed *in silico* analysis to observe if (+)-JQ1 would be predicted to inhibit canine BET proteins. First, a comparative analysis between the amino acid sequences of human (BETh) and canine (BETc) proteins and the homology of the two bromodomains of the human and canine BET proteins, respectively was performed. Each of the BET protein members evaluated, was highly conserved between human and dog, with amino acid identity ranging from 94-100% (**Table 6**), suggesting that (+)-JQ1 is able to bind to the acetylysine binding site of canine BET proteins and displace them from chromatin. Finally, an *in-silico* docking study between a (+)-JQ1 molecule and the canine BRD2, BRD3 and BRD4 proteins corroborated the binding of the inhibitor to canine BET proteins (**Figure 6**).

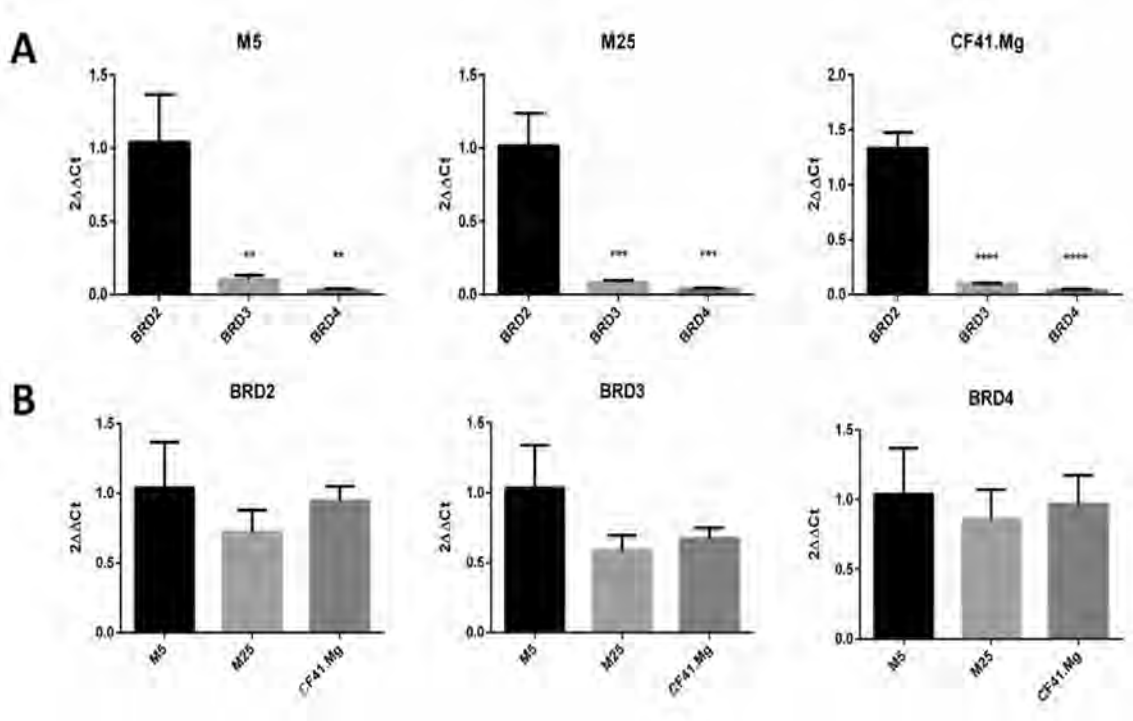


Figure 5. Gene expression analysis of BRD2, BRD3 and BRD4 in CMC cells. **(A)** The *BRD2* gene showed higher expression than the *BRD3* and *BRD4* genes in the M5, M25 and CF41 cell lines. (**B)** There was no difference in expression of the BRD2, BRD3 and BRD4 genes between M5, M25 and CF41.Mg cells. The *18S* gene was used as the housekeeping gene. (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ – One-way ANOVA followed by Tukey's multiple comparison test).

Table 5. Cycle Threshold (CT) values to *18S*, *BRD2*, *BRD3* and *BRD4* expression of M5, and M25 and CF41.Mg cells.

Samples	**CT *18S***	**CT *BRD2***	**2ΔΔCT *BRD2***	**CT *BRD3***	**2ΔΔCT *BRD3***	**CT *BRD4***	**2ΔΔCT *BRD4***
M5	13.24 ± 0.48	21.67 ± 0.10	1.03 ± 0.32	25 ± 0.15	1.03 ± 0.30	26.75 ± 0.09	1.03 ± 0.33
M25	13.10 ± 0.40	22.02 ± 0.14	0.72 ± 0.15	25.65 ± 0.25	0.58 ± 0.10	26.86 ± 0.10	0.86 ± 0.21
CF41.Mg	13.17 ± 0.34	21.69 ± 0.23	0.94 ± 0.10	25.52 ± 0.44	0.67 ± 0.07	26.76 ± 0.17	0.96 ± 0.20

Table 6. Evaluation of homology between human and canine BET proteins.

BETh	**BETc**	**Query Cover**	**Identity**
BRD2h	BRD2c	100%	98%
BRD2h (74-180)	BRD2c (74-180)	100%	100%
BRD2h (349-450)	BRD2c (349-450)	100%	100%
BRD3h	BRD3c	95%	94%
BRD3h (30-140)	BRD3c (30-140)	100%	99%
BRD3h (311-412)	BRD3c (311-412)	100%	95%
BRD4h	BRD4c	90%	97%
BRD4h (58-164)	BRD4c (58-164)	100%	99%
BRD4h (353-454)	BRD4c (353-454)	100%	100%

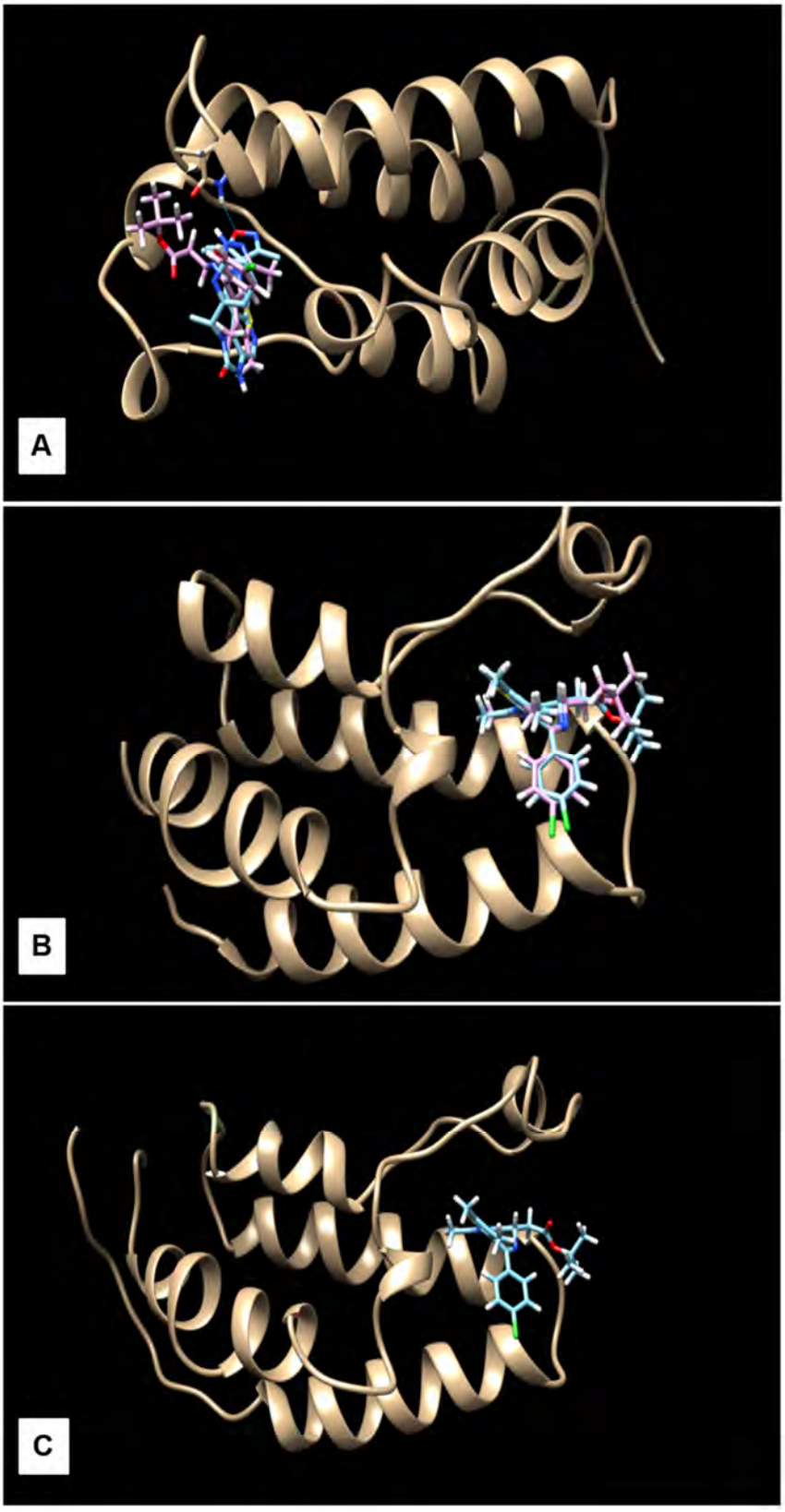


Figure 6. Docking experiments using the structure of the canine BRD2 (A), BRD3 (B) and BRD4 (C) proteins with the (+)-JQ1 ligand. The BET inhibitor (+)-JQ1 was able to bind to all canine BET proteins, specifically in the acetylated lysine binding sites.

4.4 Discussion

In the present study, we report the screening of a small-molecule epigenetic inhibitors (probes) library to modulate tumorigenicity and self-renewal phenotypes of canine mammary cancer cells. From 27 probes targeting different classes of epigenetic proteins we demonstrated that inhibition of BET proteins by (+)-JQ1 reduced the number of canine mammary colonies and tumorspheres at concentrations of 100 nM. At these low doses (+)-JQ1 did not induce apoptosis in CF41.Mg canine cells, whereas at concentration of 1 μM and above apoptotic effects were observed (**Figure 3F**; $p < 0.05$). This was accompanied by G2/M cell cycle arrest as opposed to apoptosis observed at higher concentrations. Furthermore, BET inhibition altered the expression of genes associated with self-renewal pathways including WNT, NOTCH, Hedgehog, PI3K/AKT/mTOR, EGFR and FGFR. Finally, low concentrations of (+)-JQ1 showed no cytotoxicity in CMC cells cultured in 2D *in vitro* models, suggesting BET inhibition as promising strategy to target tumorigenicity and self-renewal in CMC cells.

In this study, we showed that (+)-JQ1 targets anchorage-independent cells within canine mammary cancer cell populations. (+)-JQ1 treatment decreased colonies and tumorspheres formation by ~2-fold and ~6-fold, respectively at treatment concentrations of 100 nM. In contrast, high concentrations of (+)-JQ1 (~4 μM) reduced CF41.Mg cell numbers by 50% of when cultured in 2D *in vitro* model. Compounds that preferentially target CSCs in human breast cancer cells populations have been described previously. Two main studies have demonstrated the effects of salinomycin and metformin, substances well-known for antibacterial and antidiabetic properties, in breast cancer CSCs (GUPTA et al., 2009; HIRSCH et al., 2009). However, so far, only a few studies have demonstrated the effects of (+)-JQ1 specifically in CSCs phenotypes (VENKATARAMAN et al., 2014; HERRMANN et al., 2015) and, to our knowledge, this is the first study to demonstrate these effects in canine mammary cancer cells.

The inhibitor (+)-JQ1 inhibits specifically the family of epigenetic readers known as BET proteins (BRD2, BRD3, BRD4 and BRDT) (FILIPPAKOPOULOS et al., 2010). BRD4 is a key mediator of MYC driven transcriptional programs in c-MYC driven tumors (DELMORE et al., 2011). In human breast cancer, BRD4 plays an important role for breast tumor proliferation (NAGARAJAN et al., 2014) and BET inhibition has been shown to contribute to overcoming resistance in HER2 and hormone receptors positive

tumors (HR) (FENG et al., 2014; STUHLMILLER et al., 2015). However, triple-negative breast cancer (TNBC), the most aggressive subtype, is not commonly associated with BRD4/MYC regulation (SAHNI et al., 2016; SHU et al., 2016). These results suggest that BRD4/MYC is not the sole mechanism regulating the phenotype of breast cancer cells. Here, we show that also in canine mammary cancer cells, BET inhibition by (+)-JQ1 had no effect on the expression of MYC in cells cultured both in 2D and 3D *in vitro* models. Furthermore, when comparing expression levels of BET proteins, we found that *BRD2* showed higher expression levels compared to *BRD3* and *BRD4* in the three cell lines, suggesting that BRD2 could be a major target of (+)-JQ1 in canine mammary cancers. In fact, a recent study has shown that BET proteins could have opposing roles in epithelial-mesenchymal transition (EMT) of HR and TNBC breast cancer. BRD2 positively regulated EMT, whereas BRD3 and BRD4 repressed EMT (ANDRIEU; DENIS, 2018). However, more detailed studies are needed to elucidate the precise role of BRD2 in breast cancer.

Non-toxic doses of (+)-JQ1 decreased self-renewal and tumorigenicity and induced G2/M cell cycle arrest in CMC cells. Specifically, transcriptomic analysis by RNA-seq showed an upregulation of G2/M cell-cycle arrest genes including *BTG2*, *CCNG2* and *EGFR* genes intimately associated with cell cycle control (ROUAULT et al., 1996; HORNE et al., 1997). Previous studies showed G2/M cell-cycle arrest induced by upregulation of *CCNG2* and *BTG2* in human breast cancer cells (ZIMMERMANN et al., 2012; KATAGI et al., 2016). In contrast to the present result, some studies showed that (+)-JQ1 can increase the number of cells in G1 phase and reduce the proportion in G2/M (ASANGANI et al., 2014; ANDRIEU et al., 2016). In addition, we found *BCL2L11* to be upregulated in (+)-JQ1-treated tumorspheres. BCL2L11, also known as BIM, is a pro-apoptotic protein that leads the Bax activation, which is responsible to regulate the mitochondrial pathway to apoptosis (CZABOTAR; COLMAN; HUANG, 2009). Similar results were demonstrated in another study with B-cell Lymphoma, showing that BET proteins can induce apoptosis regulating epigenetically BCL-2 family proteins (HOGG et al., 2016).

Several lines of evidence support a role of BET proteins in the regulation of CSCs. First, BET inhibition by (+)-JQ1 had a profound impact on global gene expression in tumorspheres. DE genes were enriched in pathways related to extracellular matrix and

collagen organization, RNA and glycosaminoglycan metabolism, MET signaling and regulation of insulin-like growth factor (IGF). In particular, we observed that BET inhibition by (+)-JQ1 downregulated several genes of the IGF pathway including *CHRDL1, GPC3, SPP2, MXRA8, GAS6, BMP4, PAPPA*, and *FAM20A*. Insulin growth factor signaling is considered a critical factor for cancer stem cell survival and maintenance of the self-renewal phenotype (BENDALL et al., 2007; WANG et al., 2007; YUAN; HONG, 2017; KUO et al., 2018). In particular, *GPC3* has been suggested as a promising target for immunotherapy (HO; KIM, 2011; ORTIZ et al., 2019) and *BMP4* is a well-known factor necessary for maintenance of self-renewal, EMT and CSC phenotypes. Additionally, (+)-JQ1 decreased the expression of *ZEB2* transcription factor under 2D and 3D conditions. Recently, our group showed that CF41.Mg cells exhibit higher expression of ZEB2 in comparison with less tumorigenic CMC cells, suggesting a key role for ZEB2 in tumorigenicity and self-renewal of CMC cells (XAVIER et al., 2018). The results described in this work, open the possibility to epigenetically inhibit ZEB2 expression by targeting BET proteins in cancer cells.

Targeting self-renewal pathways is an efficient strategy to reach more tumorigenic cells, such as CSCs (BORAH et al., 2015). Nevertheless, few studies have demonstrated a direct effect of BET proteins on self-renewal-associated pathways. In human breast cancer, (+)-JQ1 reduced the number of TNBC spheroids. However, the study focused on the effect of (+)-JQ1 on TNBC response induced by hypoxia (DA MOTTA et al., 2017). Venkataraman et al have demonstrated that BET inhibition by (+)-JQ1 suppressed stem cell-associated signaling in medulloblastoma cells and inhibited medulloblastoma tumor self-renewal (VENKATARAMAN et al., 2014). Also, BET inhibition by (+)-JQ1 has been suggested to repress cell growth and modulated WNT signaling from mesenchymal stem cells without inducing apoptosis (ALGHAMDI et al., 2016).

At present, only two studies examine the role of BET proteins in canine cancer. In the first study, BRD4 was considered a novel marker and promising target in advanced mast cell neoplasms both in human and dogs (HADZIJUSUFOVIC et al., 2013). The other study presented the BET inhibitor CPI-0610 with good results and acceptable toxicity, however, dogs were used only as experimental models to preclinical trials, not as a model to describe how such proteins work on tumor progression (ALBRECHT et al., 2016). Therefore, the present study contributes to our understanding of the role of BET

proteins in the biology of CMCs, suggesting BET proteins as potential therapeutic target in CMCs.

4.5 Conclusion

In conclusion, our findings support a role for BET inhibitors in restraining self-renewal and tumorigenicity of CMC cells by altering the expression of known cancer-associated genes. This corroborates analogous studies in human cancer and highlights BET proteins as targets for the development of innovative cancer therapies for human and dogs. In addition, the results suggest that the mechanisms responsible for obtaining these phenotypes are similar in canine and human mammary cancer, underlining the validity of canine models for comparative and translational studies.

4.6 References

AHN, S. M. et al. Olig2-induced neural stem cell differentiation involves downregulation of Wnt signaling and induction of Dickkopf-1 expression. **PLoS ONE**, v. 3, n. 12, p. 1–9, 2008.

ALBRECHT, B. K. et al. Identification of a Benzoisoxazoloazepine Inhibitor (CPI-0610) of the Bromodomain and Extra-Terminal (BET) Family as a Candidate for Human Clinical Trials. **Journal of Medicinal Chemistry**, v. 59, n. 4, p. 1330–1339, 2016.

ALEKSEYENKO, A. A. et al. Ectopic protein interactions within BRD4–chromatin complexes drive oncogenic megadomain formation in NUT midline carcinoma. **Proceedings of the National Academy of Sciences**, v. 114, n. 21, p. E4184–E4192, 2017.

ALGHAMDI, S. et al. BET protein inhibitor JQ1 inhibits growth and modulates WNT signaling in mesenchymal stem cells. **Stem Cell Research and Therapy**, v. 7, n. 1, p. 1–16, 2016. Disponível em: <http://dx.doi.org/10.1186/s13287-016-0278-3>.

ALTSCHUL, S. F. et al. TLP-1997.pdf. v. 25, n. 17, p. 3389–3402, 1997.

ANDERS, S.; PYL, P. T.; HUBER, W. HTSeq-A Python framework to work with high-throughput sequencing data. **Bioinformatics**, v. 31, n. 2, p. 166–169, 2015.

ANDRIEU, G. et al. BRD4 regulates breast cancer dissemination through Jagged1/Notch1 signaling. **Cancer Research**, v. 76, n. 22, p. 6555–6567, 2016.

ANDRIEU, G. P.; DENIS, G. V. BET Proteins Exhibit Transcriptional and Functional Opposition in the Epithelial-to-Mesenchymal Transition. **Molecular Cancer Research**, v. 16, n. 4, p. 580–586, 2018.

ASANGANI, I. A. et al. Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. **Nature**, v. 510, n. 7504, p. 278–282, 23 jun. 2014. Disponível em: <http://www.nature.com/articles/nature13229>.

BATLLE, E.; CLEVERS, H. Cancer stem cells revisited. **Nature Medicine**, v. 23, n.

10, p. 1124–1134, 2017. Disponível em: <http://dx.doi.org/10.1038/nm.4409>.

BENDALL, S. C. et al. IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. **Nature**, v. 448, n. 7157, p. 1015–1021, 2007.

BENSON, D. A. et al. GenBank. **Nucleic Acids Research**, v. 43, n. D1, p. D30–D35, 2015.

BORAH, A. et al. Targeting self-renewal pathways in cancer stem cells: Clinical implications for cancer therapy. **Oncogenesis**, v. 4, n. 11, p. e177-11, 2015. Disponível em: <http://dx.doi.org/10.1038/oncsis.2015.35>.

COCOLA, C. et al. Isolation of canine mammary cells with stem cell properties and tumour-initiating potential. **Reproduction in Domestic Animals**, v. 44, n. SUPPL. 2, p. 214–217, 2009.

COCOLA, C. et al. FGF2 and EGF Are Required for Self-Renewal and Organoid Formation of Canine Normal and Tumor Breast Stem Cells. **Journal of Cellular Biochemistry**, v. 118, n. 3, p. 570–584, 2017.

CORDEIRO, Y. G. et al. Transcriptomic profile reveals molecular events associated to focal adhesion and invasion in canine mammary gland tumour cell lines. **Veterinary and Comparative Oncology**, v. 16, n. 1, p. E89–E98, mar. 2018. Disponível em: <http://doi.wiley.com/10.1111/vco.12339>.

CZABOTAR, P. E.; COLMAN, P. M.; HUANG, D. C. S. Bax activation by Bim? **Cell Death and Differentiation**, v. 16, n. 9, p. 1187–1191, 2009.

DA MOTTA, L. L. et al. The BET inhibitor JQ1 selectively impairs tumour response to hypoxia and downregulates CA9 and angiogenesis in triple negative breast cancer. **Oncogene**, v. 36, n. 1, p. 122–132, 2017.

DAWSON, M. A.; KOUZARIDES, T. Cancer epigenetics: From mechanism to therapy. **Cell**, v. 150, n. 1, p. 12–27, 2012. Disponível em: <http://dx.doi.org/10.1016/j.cell.2012.06.013>.

DELMORE, J. E. et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. **Cell**, v. 146, n. 6, p. 904–917, 2011. Disponível em: <http://dx.doi.org/10.1016/j.cell.2011.08.017>.

DOBIN, A. et al. STAR: Ultrafast universal RNA-seq aligner. **Bioinformatics**, v. 29, n. 1, p. 15–21, 2013.

FENG, Q. et al. An epigenomic approach to therapy for tamoxifen-resistant breast cancer. **Cell Research**, v. 24, n. 7, p. 809–819, 2014. Disponível em: <http://dx.doi.org/10.1038/cr.2014.71>.

FILIPPAKOPOULOS, P. et al. Selective inhibition of BET bromodomains. **Nature**, v. 468, n. 7327, p. 1067–1073, 2010. Disponível em: <http://dx.doi.org/10.1038/nature09504>.

FOLMER, O. et al. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. **Molecular marine biology and biotechnology**, v. 3, n. 5, p. 294–299, 1994.

GARCÍA-ALCALDE, F. et al. Qualimap: Evaluating next-generation sequencing

alignment data. **Bioinformatics**, v. 28, n. 20, p. 2678–2679, 2012.

GUPTA, P. B. et al. Identification of Selective Inhibitors of Cancer Stem Cells by High-Throughput Screening. **Cell**, v. 138, n. 4, p. 645–659, 2009. Disponível em: <http://dx.doi.org/10.1016/j.cell.2009.06.034>.

HADZIJUSUFOVIC, E. et al. Bromodomain-Containing Protein 4 (BRD4): A Novel Marker and Drug Target Expressed In Neoplastic Cells In Advanced Mast Cell Neoplasms. **Blood**, v. 122, n. 21, p. 3747 LP – 3747, 15 nov. 2013. Disponível em: <http://www.bloodjournal.org/content/122/21/3747.abstract>.

HERRMANN, H. et al. Small-molecule inhibition of BRD4 as a new potent approach to eliminate leukemic stem- and progenitor cells in acute myeloid leukemia (AML). **Oncotarget**, v. 3, n. 12, p. 1588–1599, 2015.

HIRSCH, H. A. et al. Metformin Selectively Targets Cancer Stem Cells, and Acts Together with Chemotherapy to Block Tumor Growth and Prolong Remission. **Cancer Research**, v. 69, n. 19, p. 7507–7511, 1 out. 2009. Disponível em: <http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-09-2994>.

HO, M.; KIM, H. Glypican-3: A new target for cancer immunotherapy. **European Journal of Cancer**, v. 47, n. 3, p. 333–338, 2011.

HOGG, S. J. et al. BET Inhibition Induces Apoptosis in Aggressive B-Cell Lymphoma via Epigenetic Regulation of BCL-2 Family Members. **Molecular Cancer Therapeutics**, v. 15, n. 9, p. 2030–2041, 2016.

HORNE, M. C. et al. Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. **Journal of Biological Chemistry**, v. 272, n. 19, p. 12650–12661, 1997.

JENSEN, L. J. et al. STRING 8 - A global view on proteins and their functional interactions in 630 organisms. **Nucleic Acids Research**, v. 37, n. SUPPL. 1, p. 412–416, 2009.

JONES, P. A.; BAYLIN, S. B. The Epigenomics of Cancer. **Cell**, v. 128, n. 4, p. 683–692, fev. 2007. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0092867407001274>.

JOSLING, G. A. et al. The role of bromodomain proteins in regulating gene expression. **Genes**, v. 3, n. 2, p. 320–343, 2012.

KATAGI, A. et al. Inhibitory effect of isoamericanol A from Jatropha curcas seeds on the growth of MCF-7 human breast cancer cell line by G2/M cell cycle arrest. **Heliyon**, v. 2, n. 1, 2016. Disponível em: <http://dx.doi.org/10.1016/j.heliyon.2015.e00055>.

KEARSE, M. et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. **Bioinformatics**, v. 28, n. 12, p. 1647–1649, 2012.

KIMURA, M. Evolutionary Rates models. **J. Mol. Evol**, v. 16, n. 1330, p. 111–120, 1980.

KUNTZ ID, BLANEY JM, OATLEY SJ, LANGRIDGE R, F. TE. A geometric approach to macromolecule-ligand interactions. **J Mol Biol**, v. 161, n. 2, p. 269–288,

1982.

KUO, Y. C. et al. IGF-1R Promotes Symmetric Self-Renewal and Migration of Alkaline Phosphatase+ Germ Stem Cells through HIF-2α-OCT4/CXCR4 Loop under Hypoxia. **Stem Cell Reports**, v. 10, n. 2, p. 524–537, 2018. Disponível em: <https://doi.org/10.1016/j.stemcr.2017.12.003>.

LI, H. et al. The Sequence Alignment/Map format and SAMtools. **Bioinformatics (Oxford, England)**, v. 25, n. 16, p. 2078–9, ago. 2009.

LI, N. et al. BET bromodomain inhibitor JQ1 preferentially suppresses EBV-positive nasopharyngeal carcinoma cells partially through repressing c-Myc article. **Cell Death and Disease**, v. 9, n. 7, p. 1–12, 2018. Disponível em: <http://dx.doi.org/10.1038/s41419-018-0789-1>.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. **Methods**, v. 25, n. 4, p. 402–408, 2001.

MERTZ, J. A. et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. **Proceedings of the National Academy of Sciences**, v. 108, n. 40, p. 16669–16674, 2011.

MOUSTAKAS, D. T. et al. Development and validation of a modular, extensible docking program: DOCK 5. **Journal of Computer-Aided Molecular Design**, v. 20, n. 10–11, p. 601–619, 2006.

MULLER, S.; FILIPPAKOPOULOS, P.; KNAPP, S. Bromodomains as therapeutic targets. **Expert Reviews in Molecular Medicine**, v. 13, n. September 2011, p. e29, 2011. Disponível em: <http://www.journals.cambridge.org/abstract_S1462399411001992>.

NAGARAJAN, S. et al. Bromodomain Protein BRD4 Is Required for Estrogen Receptor-Dependent Enhancer Activation and Gene Transcription. **Cell Reports**, v. 8, n. 2, p. 460–469, 2014.

NUMATA, M.; KLEIN GELTINK, R. I.; GROSVELD, G. C. Establishment of a transgenic mouse to model ETV7 expressing human tumors. **Transgenic Research**, v. 28, n. 1, p. 115–128, 2019. Disponível em: <https://doi.org/10.1007/s11248-018-0104-z>.

OCAÑA, A.; NIETO-JIMÉNEZ, C.; PANDIELLA, A. BET inhibitors as novel therapeutic agents in breast cancer. **Oncotarget**, v. 8, n. 41, p. 71285–71291, 2017.

ORTIZ, M. V. et al. Immunotherapeutic Targeting of GPC3 in Pediatric Solid Embryonal Tumors. **Frontiers in Oncology**, v. 9, n. February, p. 1–8, 2019.

PALUMBI, S. et al. the Simple Guide To Pcr. v. 96822, n. 808, p. 1–45, 2002.

PAOLONI, M.; KHANNA, C. Translation of new cancer treatments from pet dogs to humans. **Nature Reviews Cancer**, v. 8, n. 2, p. 147–156, 2008.

PETTERSEN, E. F. et al. UCSF Chimera--a visualization system for exploratory research and analysis. **Journal of computational chemistry**, v. 25, n. 13, p. 1605–12, 2004. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15264254>.

QUEIROGA, F. L. et al. Canine mammary tumours as a model to study human breast cancer: most recent findings. **In vivo (Athens, Greece)**, v. 25, n. 3, p. 455–465, 2011.

RIBBLE, D. et al. A simple technique for quantifying apoptosis in 96. **BMC Biotechnology**, v. 7, p. 1–7, 2005.

RICHARDS, F. M. Areas, Volumes, Packing, and Protein Structure. **Annual Review of Biophysics and Bioengineering**, v. 6, n. 1, p. 151–176, 1977. Disponível em: <http://dx.doi.org/10.1146/annurev.bb.06.060177.001055>.

ROBINSON, M. D.; MCCARTHY, D. J.; SMYTH, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. **Bioinformatics**, v. 26, n. 1, p. 139–140, 2009.

RODRIGUEZ, R. M. et al. Aberrant epigenetic regulation of bromodomain Brd4 in human colon cancer. **Journal of Molecular Medicine**, v. 90, n. 5, p. 587–595, 2012.

ROMAGNOLO, D. F. et al. Epigenetics of breast cancer: Modifying role of environmental and bioactive food compounds. **Molecular Nutrition & Food Research**, v. 60, n. 6, p. 1310–1329, jun. 2016. Disponível em: <http://doi.wiley.com/10.1002/mnfr.201501063>.

ROUAULT, J.-P. et al. Identification of BTG2, an antiproliferative p53–dependent component of the DNA damage cellular response pathway. **Nature Genetics**, v. 14, n. 4, p. 482–486, dez. 1996. Disponível em: <http://www.nature.com/articles/ng1296-482>.

RYBICKA, A.; KRÏ¿½L, M. Identification and characterization of cancer stem cells in canine mammary tumors. **Acta Veterinaria Scandinavica**, v. 58, n. 1, p. 1–7, 2016.

SAHNI, J. M. et al. Bromodomain and extraterminal protein inhibition blocks growth of triple-negative breast cancers through the suppression of Aurora kinases. **Journal of Biological Chemistry**, v. 291, n. 45, p. 23756–23768, 2016.

SAITOU, N.; NEI, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. **Molecular Biology and Evolution**, v. 4, n. 4, p. 406–425, 1987.

SCHEER, S. et al. A chemical biology toolbox to study protein methyltransferases and epigenetic signaling. **Nature Communications**, v. 10, n. 1, p. 1–14, 2019. Disponível em: <http://dx.doi.org/10.1038/s41467-018-07905-4>.

SHU, S. et al. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. **Nature**, v. 529, n. 7586, p. 413–417, 2016. Disponível em: <http://dx.doi.org/10.1038/nature16508>.

SINGER, E. et al. Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma. **Cell Death and Disease**, v. 6, n. 1, p. e1601-11, 2015. Disponível em: <http://dx.doi.org/10.1038/cddis.2014.566>.

STUHLMILLER, T. J. et al. Inhibition of lapatinib-induced kinome reprogramming in ERBB2-positive breast cancer by targeting BET family bromodomains. **Cell Reports**, v. 11, n. 3, p. 390–404, 2015. Disponível em: <http://dx.doi.org/10.1016/j.celrep.2015.03.037>.

SZKLARCZYK, D. et al. STRING v11: Protein-protein association networks with

increased coverage, supporting functional discovery in genome-wide experimental datasets. **Nucleic Acids Research**, v. 47, n. D1, p. D607–D613, 2019.

TANIGUCHI, Y. The bromodomain and extra-terminal domain (BET) family: Functional anatomy of BET paralogous proteins. **International Journal of Molecular Sciences**, v. 17, n. 11, 2016.

VALLONE, P. M.; BUTLER, J. M. AutoDimer: A screening tool for primer-dimer and hairpin structures. **BioTechniques**, v. 37, n. 2, p. 226–231, 2004.

VENKATARAMAN, S. et al. Inhibition of BRD4 attenuates tumor cell self-renewal and suppresses stem cell signaling in MYC driven medulloblastoma. **Oncotarget**, v. 5, n. 9, p. 2355–71, 2014. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4058011&tool=pmcentrez&rendertype=abstract>.

WAHL, G. M.; SPIKE, B. T. Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. **npj Breast Cancer**, v. 3, n. 1, p. 1–12, 2017. Disponível em: <http://dx.doi.org/10.1038/s41523-017-0012-z>.

WANG, L. et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. **Blood**, v. 110, n. 12, p. 4111–4119, 2007.

WEISWALD, L. B.; BELLET, D.; DANGLES-MARIE, V. Spherical Cancer Models in Tumor Biology. **Neoplasia (United States)**, v. 17, n. 1, p. 1–15, 2015. Disponível em: <http://dx.doi.org/10.1016/j.neo.2014.12.004>.

WU, B. et al. Epigenetic drug library screening identified an LSD1 inhibitor to target UTX-deficient cells for differentiation therapy. **Signal Transduction and Targeted Therapy**, v. 4, n. 1, p. 1–10, 2019. Disponível em: <http://dx.doi.org/10.1038/s41392-019-0040-2>.

XAVIER, P. L. P. et al. ZEB1 and ZEB2 transcription factors are potential therapeutic targets of canine mammary cancer cells. **Veterinary and Comparative Oncology**, v. 16, n. 4, p. 596–605, 2018.

XU, L. et al. Targetable BET proteins- and E2F1-dependent transcriptional program maintains the malignancy of glioblastoma. **Proceedings of the National Academy of Sciences**, v. 115, n. 22, p. E5086–E5095, 2018.

YANG, Z. et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. **Molecular Cell**, v. 19, n. 4, p. 535–545, 2005.

YE, J. et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. **BMC bioinformatics**, v. 13, p. 134, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22708584%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3412702>.

YUAN, Y.; HONG, Y. Medaka insulin-like growth factor-2 supports self-renewal of the embryonic stem cell line and blastomeres in vitro. **Scientific Reports**, v. 7, n. 1, p. 1–11, 2017. Disponível em: <http://dx.doi.org/10.1038/s41598-017-00094-y>.

ZHANG, Y. et al. The roles of ZEB1 in tumorigenic progression and epigenetic modifications. **Biomedicine and Pharmacotherapy**, v. 110, n. September 2018, p.

400–408, 2019. Disponível em: <https://doi.org/10.1016/j.biopha.2018.11.112>.

ZIMMERMANN, M. et al. Elevated cyclin G2 expression intersects with DNA damage checkpoint signaling and is required for a potent G2/M checkpoint arrest response to doxorubicin. **Journal of Biological Chemistry**, v. 287, n. 27, p. 22838–22853, 2012.

ZUBER, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. **Nature**, v. 478, n. 7370, p. 524–528, out. 2011.

5. Chapter 4: Dual HDAC-BET Proteins Inhibition by TW09 Hold Promise for Human Breast Cancer

Pedro L. P. Xavier [1]; Tim Weiser [2]; Romain Lucas [2]; Stefan Knapp [2]; Heidge Fukumasu [1]; Susanne Müller [2]

[1] Laboratory of Comparative and Translational Oncology (LOCT), Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, Brazil

[2] Structural Genomics Consortium and Institute of Pharmaceutical Chemistry, Buchmann Institute for Molecular Life Sciences, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

Abstract

Mammary tumors are the most frequent type of cancer in women as in female dogs. Besides that, the cells share similar features and can be used for comparative studies and screening of antineoplastic agents. Epigenetic targets are highlighted as potential and innovative therapy against cancer. Both histone deacetylases (HDACs) and bromodomain and extraterminal proteins (BET) inhibitors have been shown to be efficient in types of cancer such as lymphoma, leukemia, and NUT midline carcinomas. In breast cancer, few studies have observed good effects of these inhibitors. Furthermore, when applying individually, some issues may arise, including incomplete efficacy and drug resistance. Thus, new strategies to overcome these limitations are necessary and one of the possibilities is to test the combined effect of HDAC-BET inhibition using a dual inhibitor. Here, we test a new dual HDAC-BET inhibitor (TW09), designed based on (+)-JQ1 (BET inhibitor) and CI994 (HDAC inhibitor) structures. We determined the effects of TW09 on both Hormone Receptor-positive cell lines (HR-positive), and triple-negative breast cancer (TNBC) cell lines. To determine the effects on cell viability, cells were treated with (+)-JQ1 (BET inhibitor), CI994 (HDAC inhibitor), (+)-JQ1 + CI994, and TW09 (dual HDAC-BET inhibitor) with serial dilutions and, after 72h, cell viability was tested using MTT assay. The triple-negative cell line, MDA-MB-231, was more resistant to all inhibitors. TW09 reduces cell viability in a dose-dependent manner and IC_{50} was more potent compared to (+)-JQ1 and CI994 individually in both breast cancer cell lines (BC), but not exhibited significant difference in comparison with (+)-JQ1 + CI994. TW09 also decrease number of primary tumorspheres. In addition, TW09 was able to inhibit the formation of secondary tumorspheres, even after removal of the treatment during

dissociation of primary tumorspheres, pointing to an epigenetic effect. We also observed the effects of TW09 in established tumorspheres, after 2 days of growth. In this case, concentrations higher than 1 μM of TW09 were necessary to decrease the number of tumorspheres. Therefore, we observed good effects of the new dual HDAC-BET inhibitor TW09 in breast cancer cells using *in vitro* assays, elucidating a promising new treatment strategy for breast cancer.

Keywords: breast Cancer, BET proteins, epigenetics, HDACs, TW09

5.1 Introduction

Epigenetic mechanisms play a prominent role in gene expression regulation and cellular states. Consequently, epigenetic aberrations are associated with development and progression of most types of cancer (JONES; BAYLIN, 2007). Epigenetic targets can be categorized into 3 main classes: writers (*e.g.* Histone methyltransferases), erasers (*e.g.* histone deacetylases) and readers (*e.g.* Bromodomain-containing proteins). Therefore, therapies that target these epigenetic proteins classes could have a major clinical impact against cancer (DAWSON; KOUZARIDES, 2012).

Histone acetylation and deacetylation are one of the main histone post-translational modification and play a key role in regulating the transcriptional machinery (CLAYTON; HAZZALIN; MAHADEVAN, 2006). Histone acetylation, induces the neutralization of the histone tail positive charge, resulting in an open state of the chromatin, permissive to the recruitment of the transcriptional machinery. The process is regulated by two groups of enzymes called histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC proteins are epigenetic erasers and are responsible for removing acetyl groups from lysine residues of specific histones, as well as non-histone proteins, while HATs catalyses the opposite reaction (YANG; SETO, 2007). HDAC inhibitors (HDACi) have been widely studied in both pre-clinically and clinically in oncology and are considered good candidates for cancer therapy (BUTLER et al., 2002; RICHON, 2006; GLOZAK; SETO, 2007; LI; ZHU, 2014). As single agents, HDACi have shown therapeutic efficacy in multiple cancer types including lymphoma (GUO; ZHANG, 2012), acute myeloid leukemia (SCHAEFER et al., 2009), multiforme glioblastoma, (GALANIS et al., 2009) and non-small cell lung cancer (KOMATSU et al., 2006). However, HDACi has proven less successful for the treatment of breast cancer (BORBELY et al., 2015).

The bromodomain and extraterminal proteins (BET) family of proteins are epigenetic readers and is composed of four members: BRD2, BRD3, BRD4 and BRDT (FILIPPAKOPOULOS et al., 2010). These proteins possess two N-terminal bromodomains that recognize, “read” acetyl-lysine residues and recruit a complex of regulatory proteins including positive transcription elongation factor b (P-TEFb) (YANG et al., 2005; MULLER; FILIPPAKOPOULOS; KNAPP, 2011; JOSLING et al., 2012). BET proteins have been shown to play key roles in cancer and are considered attractive therapeutic targets. Several small molecules inhibitors of BET proteins, including (+)-JQ1 and iBETs, exhibit anti-neoplastic effects in acute myeloid leukemia (ZUBER et al., 2011), multiple myeloma (MERTZ et al., 2011), NUT midline carcinoma (ALEKSEYENKO et al., 2017), colon cancer (RODRIGUEZ et al., 2012) and breast cancer (OCAÑA; NIETO-JIMÉNEZ; PANDIELLA, 2017). However, strategies to augment the efficacy of these BET inhibitors are still few (RAINA et al., 2016; TANAKA et al., 2016).

The co-inhibition of HDACs and BET proteins, has shown therapeutic efficacy in some types of cancer including lymphoma, melanoma and urothelial carcinoma (BHADURY et al., 2014; HEINEMANN et al., 2015; HÖLSCHER et al., 2018). In breast cancer, a study published by Borbely et al., 2015 (BORBELY et al., 2015), observed a good synergistic effect of co-treatment using (+)-JQ1 (BET inhibitor) and mocetinostat (HDAC inhibitor), resulting in a decrease of breast cancer cells viability. However, further studies designed with different approaches should be performed to demonstrate a clinical impact of the HDAC-BET inhibition in breast cancer. In this sense, the new dual HDAC-BET inhibitor, TW09, has been developed (LASZIG et al., 2020) . Thus, the aim of this study was to test the effects of the TW09 in breast cancer cell lines using 2D and 3D culture models. We showed that HDAC-BET inhibition by TW09 is a promising strategy to treat breast cancer cells decreasing cell viability in a dose-dependent manner and the number of tumorspheres, suggesting effects on tumorigenicity and self-renewal potential of breast cancer cells.

5.2 Material and Methods

5.2.1 Cell Lines

Two breast cancer cell lines and one canine mammary cancer cell were used: the HR-positive cell line, MCF7 and the triple-negative breast cancer cell line, MDA-MB-231. These cells were kindly provided by Dr. Susanne Müller (Institute of Pharmaceutical

Chemistry, Buchmann Institute for Molecular Life Sciences, Johann Wolfgang Goethe University, Frankfurt am Main, Germany). Cell lines were cultured in 75 cm^2 flasks at 37°C and 5% of CO_2 with DMEM - high glucose (Sigma-Aldrich, USA) supplemented with 10% of FBS and 1% of penicillin/streptomycin. CF41.Mg was cultured with DMEM-F12 supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Passaging was performed when cells were 85% confluent. Culture evolution was evaluated daily by optical microscopy.

5.2.2 Real-Time PCR (qPCR)

Total RNA was extracted from cells using Trizol® following the manufacturer's instructions. Gene expression analyses were performed by real-time PCR using a StepOne System (Thermo Fisher Scientific, USA). Specific primers sequence for *RPLP0*, *BRD4*, and *c-Myc* used in this project are described in Montenegro et al. 2016 (MONTENEGRO et al., 2016). Specific primers for *HDAC1* were designed with Primer-BLAST (YE et al., 2012) and the absence of dimers and hairpins were verified using AutoDimer software (VALLONE; BUTLER, 2004). Primers were also analyzed by in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) to confirm specificity. Primer sequences are reported in **Supplementary Table S1, Appendix D**. PCR reactions were carried out using Power SYBR Green Master Mix in a final volume of 10 µl. Conditions for quantitative PCR were as follows: 95 °C for 10 min; 40 cycles at 95°C for 15s for denaturation, 60°C for 1 min for annealing/extend; melt curve analysis was performed at 95 °C for 15 s and 60 °C for 60 s. The housekeeping gene to which expression levels of the other genes were normalized was *RPLP0* and the analysis of relative gene expression data was performed according to the ΔΔCt method (LIVAK; SCHMITTGEN, 2001). To observe the effects of TW09 in the *c-Myc* expression cells were treated with 0.5 µM of TW09 for 6h and then the total RNA and PCR reactions were performed as described above. Experiments were performed twice and in biological triplicates. All the reagents were purchased from Thermo Fisher Scientific.

5.2.3 Cell viability, IC_{50} determination, and Inhibitors Synergy

TW09, (+)-JQ1, and CI994 (**Supplementary Table S2, Appendix D**) were dissolved in DMSO to a concentration of 50 mM. MCF7 and MDA-MB-231 cells were seeded at 3.000 cells/well in 96 well plates containing 200 µl of supplemented media as described above. After 24 hours, media was replaced by new culture media containing

different concentrations of the compounds, ranging from 10 μM to 0.15625 μM. The compounds were added in six replicates per concentration and the experiment was performed in biological triplicates. After 72h, 10 μl of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT - 5 mg/mL) was added to each well and formazan crystals were produced over a 1h incubation period. One hundred μl of DMSO were added to dissolve crystals. Optical density at 540 nm was measured in a Fluorstar Optima (BMG Labtech, Germany). The concentration of compounds resulting in IC_{50} was calculated for each cell line using a nonlinear regression test performed in GraphPad Prism (Version 6.0 for Windows, GraphPad Software, USA). For determination of inhibitors synergism, (+)-JQ1 and CI994 were used in constant dose ratios (1:1). For each cell line, six different combinations of concentrations were used and then interaction between (+)-JQ1 and CI994 was calculated by the Combination Index (CI) according Chou-Talalay method using CompuSyn software (CHOU; TALALAY, 1983; MARTIN et al., 2010). CI values indicate synergism when CI < 1, additivity when =1 and antagonism when CI > 1. We also calculated synergism using HSA score using SynergyFinder Software (IANEVSKI et al., 2017). In this case, synergism is determined when HSA score >10, additivity when score is between 10 and -10, and antagonism when scores are < -10.

5.2.4 Tumorspheres formation assay

Single cells of MCF7 and T-47D cell lines were seeded into an ultra-low attachment surface 24-well plate (Corning) at the density of 8×10^2 suspended in 0.5 mL of serum-free DMEM supplemented with 1x B27 (Thermo Fisher Scientific, USA), 20 ng/ml of EGF (PrepoTech, USA), 10 ng/ml of FGF (PrepoTech, USA), 5 μg/ml of bovine insulin (Sigma-Aldrich, USA), 4 μg/ml of heparin and 1% penicillin/streptomycin. Cells were treated with DMSO (control) or different concentrations of TW09 (0.1 μM, 0.25 μM, and 0.5 μM) for 4 days and then the number of tumorspheres was evaluated. To generate secondary tumorspheres, primary tumorspheres were dissociated with trypsin and single cells in the same density were seeded to grow for more 4 days in two different conditions: 1) we maintained the concentrations of the TW09 during the growth of the secondary tumorspheres; 2) we removed the TW09 treatment during the growth of secondary tumorspheres. The number of tumorspheres was evaluated after 4 days. Finally, we tested the effects of TW09 in established tumorspheres. For this, we allow the tumorspheres to grow for 2 days and then we treated with different concentrations of

TW09 for 48h. Pictures were taken with optical microscopy (Axio Observer.Z1, Zeiss, Germany).

5.2.5 Cell Death Assay

Cell death was measured by Acridine orange + ethidium bromide (AO/EB) assay. BC cells were seeded in 6-well plates and after 24 h, cells were treated with DMSO, CI994, (+)-JQ1, (+)-JQ1 + CI994, and TW09 at a final concentration of 2.5 µM for 72h. After, a dye mix containing 100 µg/ml of acridine orange and 100 µg/ml of ethidium bromide was added to cells and observed for fluorescence emission using ZEISS—Axio Vert A1 with a camera Axio Can 503 attached using a 520 nm and 620 nm wavelength filter for green and red colors, respectively (ZEISS). Analyses were performed in triplicate, counting a minimum of 100 total cells each.

5.2.6 Cell Death Analysis in Tumorspheres

In normal cells, phosphatidylserine (PS) residues are found in the inner membrane of the cytoplasmic membrane. However, during apoptosis, the PS residues are translocated in the membrane and are exposed on the outer leaflet of the membrane. Annexin-V is a specific PS-binding protein that can be used to detect, in general, early apoptotic cells. In addition, Yo-Pro3 is a dye which penetrate apoptotic cells because of permeability changes associated with the loss of plasma membrane integrity. Thus, we used both to observe cell death in TW09 tumorspheres. Control and TW09-treated tumorspheres were stained with Hoechst 33342 (1 µM) (Thermo Fisher Scientific, USA), Yo-Pro3 (1 µM) (Thermo Fisher Scientific, USA) and Annexin V (0.3 µl per well) (Thermo Fisher Scientific, USA). Dyes were added directly in the low-adherent wells and after 1 hour of incubation, tumospheres were visualized in the Zeiss LSM780 (Zeiss, Germany).

5.2.7 Caspase-3 staining of the tumorspheres

Tumorspheres treated with different concentrations of the TW09 were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature (RT). Tumorspheres were rinsed three times and permeabilizated with Triton X-100 + Tween-20 for 15 min at RT. Then, tumorspheres were blocked with 5% BSA for 1h at RT and later were incubated with specific primary antibody to Caspase-3 (active) (Asp175, rabbit, polyclonal, Cell Signalling) diluted in blocking solution (1:400) at 37°C overnight.

Tumorspheres were rinsed three times and incubated at 37°C for 4h with the corresponding secondary antibody at a dilution of 1:200. Finally, tumorspheres were incubated with Hoechst 33342 (1:1000) for 10 min at RT. Tumorspheres were observed for confocal microscopy using the Zeiss LSM780 and pictures were taken using the software ZEN. *Hoechst* 33342 was excited by 35 ms exposure; Ex 360-400 nm/Em 410-480 nm, Caspase-3 by 35 ms exposure; Ex 560-580 nm/Em 650-760 nm.

5.2.8 Statistical Analysis

The IC50 was calculated using nonlinear regression test. Gene expression and tumorsphere formation were analyzed by one-way ANOVA with post hoc Tukey and Dunnett. Unpaired T-test was used for gene expression analysis of non-treated and TW09-treated cells.

5.3 Results

5.3.1 Expression of BRD4 and HDAC1 in the breast cancer cell lines

BET family of proteins are epigenetic readers, recognizing acetylated chromatin. It consists of 4 members: BRD2, BRD3, BRD4, and BRDT (MULLER; FILIPPAKOPOULOS; KNAPP, 2011). Meanwhile, class I HDACs are epigenetic erasers and remove chromatin acetylation (HABERLAND; MONTGOMERY; OLSON, 2011). Both proteins develop a key role in cancer and many strategies have been developed to inhibit these proteins (MANZOTTI; CIARROCCHI; SANCISI, 2019). In order to confirm the mRNA expression of *BRD4* and *HDAC1* in the breast cancer cells, we performed qPCR analysis. *BRD4* and *HDAC1* were highly expressed in both MCF7 and MDA-MB-231 cells (**Table 1**). Highest expression of BRD4 and HDAC1 was found in MDA-MB-231 cell line (**Figure 1**).

Table 1. Cycle Threshold (CT) values to *BRD4 and HDAC1* expression of cell lines.

Cell line	CT *RPLP0*	CT *BRD4*	ΔCT BRD4	2ΔΔCT *BRD4*	CT HDAC1	ΔCT HDAC1	2ΔΔCT HDAC1
MCF7 (HR-positive)	16.67 ± 0.67	19.44 ± 0.18	2.77 ± 0.53	0.78 ± 0.31	20.82 ± 0.35	4.15 ± 0.44	0.24 ± 0.07
MDA-MB-231 (TNBC)	17.88 ± 0.37	19.18 ± 0.06	1.3 ± 0.4	2.13 ± 0.63	19.64 ± 0.18	1.76 ± 0.39	1.26 ± 0.36

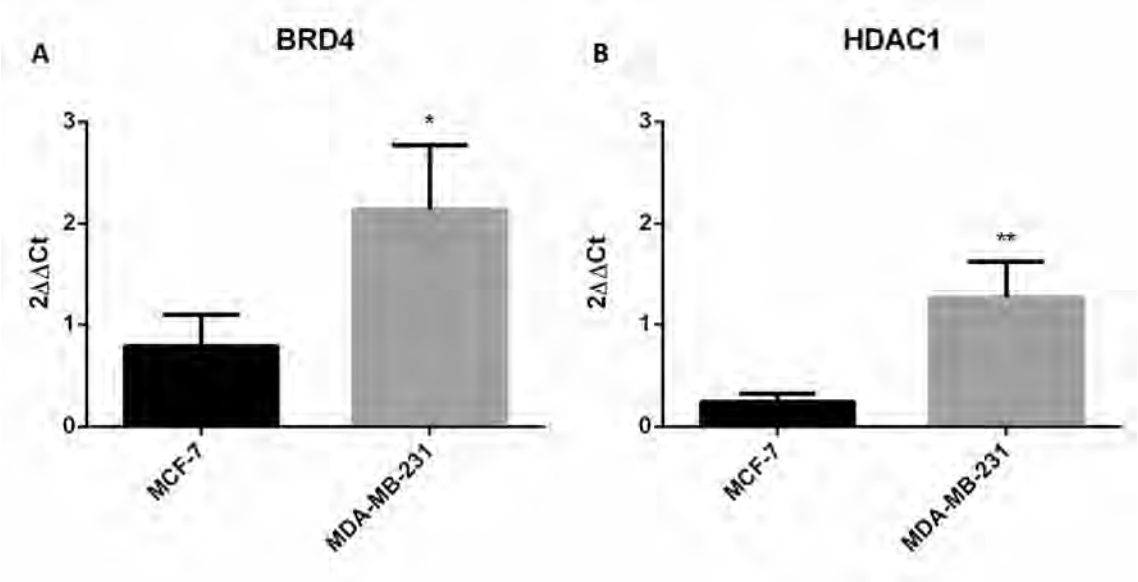


Figure 1. Gene expression analysis of BRD4 and HDAC1. MDA-MB-231 cells showed higher expression of BRD4 (**A**) and HDAC1 (**B**) in comparison with MCF7 cells (*$p < 0.05$; ** $p < 0.01$ – Unpaired T-test).

5.3.2 TW09 induces cell viability reduction and cell death in BC cells

To compare the effect of dual BET-HDAC inhibition in BC cell viability, we first determined the synergism of (+)-JQ1 and CI994 in both HR-positive and TNBC cells. On MCF7 cells, co-treatment of (+)-JQ1 + CI994 induced synergistic effects in the most of concentrations tested (**Table 2**; **Figure 2A**), exhibiting CI < 1. In addition, HAS synergy score of (+)-JQ1 + CI994 was higher than 10, confirming synergism (**Figure 2B**). However, co-treatment of (+)-JQ1 + CI994 in MDA-MB-231 cell line induced synergistic effects only in the higher concentration tested (10 μM) (**Table 3**; **Figure 2C**) and, HAS synergy score indicated a slight additive effect in these cells (**Figure 2D**). Next, we observed TW09 effects in comparison with (+)-JQ1, CI994, and (+)-JQ1 + CI994 co-treatment on cell viability. After 72h of treatment, we detected that triple-negative MDA-MB-231 cells were more resistant in comparison with MCF7 cells to all inhibitors tested (**Figure 3**). TW09 showed the greatest inhibition of cell viability in a dose-dependent manner and the most potent IC_{50} ($p < 0.05$) when compared to (+)-JQ1 and CI994 tested individually on both BC cells (**Figure 3A-B; Supplementary Figure 1 and 2**, **Appendix D**; $p < 0.05$). Although did not exhibit a significant lower IC_{50} in comparison with (+)-JQ1 + CI994 co-treatment, TW09 resulted in stronger reduction of cell viability in the most of doses tested in MCF7 cells excepting in the lowest and highest doses (0.15625

and 10 μM) (**Figure 3C**; $p < 0.05$). In MDA-MB-231 cells, TW09 was more efficient than (+)-JQ1 + CI994 co-treatment in the highest doses tested (1.25, 2.5, 5, and 10 μM) (**Figure 2D**; $p < 0.05$). Regarding cell death, in an equimolar concentration (2.5 μM), TW09 increased cell death in comparison with (+)-JQ1 and CI994 individually assessed by AO/EB assay (**Figure 4F and 5F**; $p < 0.05$). However, TW09 showed no significant difference inducing cell death in comparison with (+)-JQ1 + CI994 co-treatment (**Figure 4F and 5F**).

Table 2. Fa and CI values for different doses of (+)-JQ1 + CI994 co-treatment in MCF7 cells. Fa values are distributed from 0 to 1 and represents the inhibition effects in MCF7 cells. Fa values close to 1 indicate greater inhibition. CI < 1 indicate synergism, CI = 1 indicate additivity and CI > 1 antagonism.

Total concentration (μM)	Fa CI994	Fa (+)-JQ1	Fa (+)-JQ1 + CI994	CI value
10	0.76863	0.77205	0.91621	0.18927
5	0.64055	0.74806	0.83969	0.19899
2.5	0.44059	0.73626	0.79238	0.18275
1.25	0.23219	0.70519	0.75001	0.20873
0.625	0.13190	0.69598	0.68962	0.45662
0.3125	0.06423	0.64475	0.58729	2.97776

Table 3. Fa and CI values for different doses of (+)-JQ1 + CI994 co-treatment in MDA-MB-231 cells. Fa values are distributed from 0 to 1 and represents the inhibition effects in MDA-MB-231 cells. Fa values close to 1 indicate greater inhibition. CI < 1 indicate synergism, CI = 1 indicate additivity and CI > 1 antagonism.

Total concentration (μM)	Fa CI994	Fa (+)-JQ1	Fa (+)-JQ1 + CI994	CI value
10	0.64266	0.65390	0.69825	0.64015
5	0.31423	0.60211	0.53913	1.53963
2.5	0.13361	0.57806	0.47054	1.59936
1.25	0.0.05851	0.53674	0.41997	1.40860
0.625	0.03946	0.47949	0.32292	2.30686
0.3125	0.02173	0.33529	0.24549	3.49041

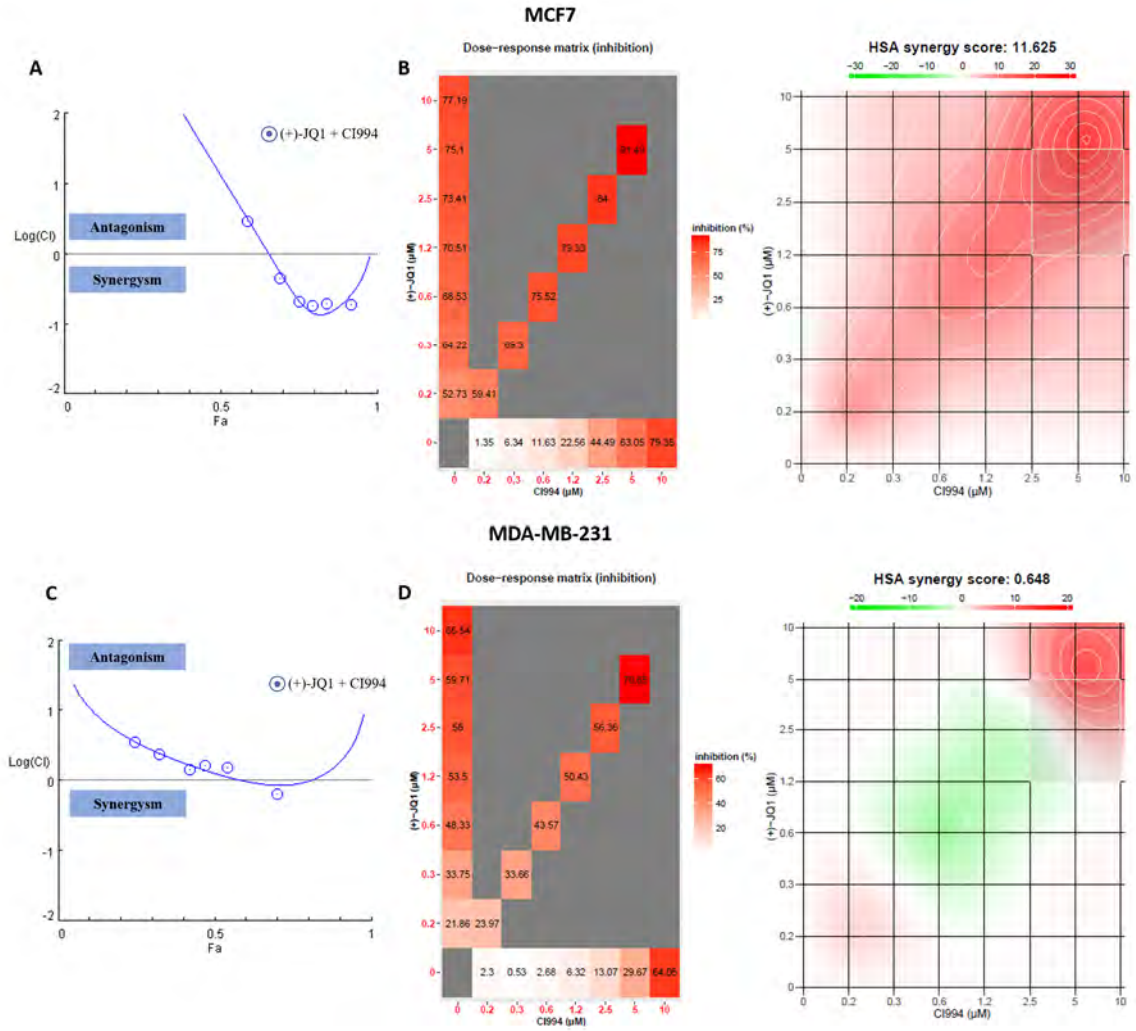


Figure 2. Synergism effects of (+)-JQ1 and CI994 in BC cells. Co-treatment of (+)-JQ1 + CI994 cells exhibited synergism in MCF7 cells, exhibiting Combination Index (CI) < 1 (Log(CI) < 0) according Chou-Talalay method (**A**) and HSA score > 10 using SynergyFinder softwate (**B**). In MDA-MB-231 cells, only the higher concentration of (+)-JQ1 + CI994 (10 μM) showed synergism. The most of dose points presented CI > 1 (Log(CI) > 0) (**C**) and HAS score nearly zero (**D**), pointing to a slightly additive effect.

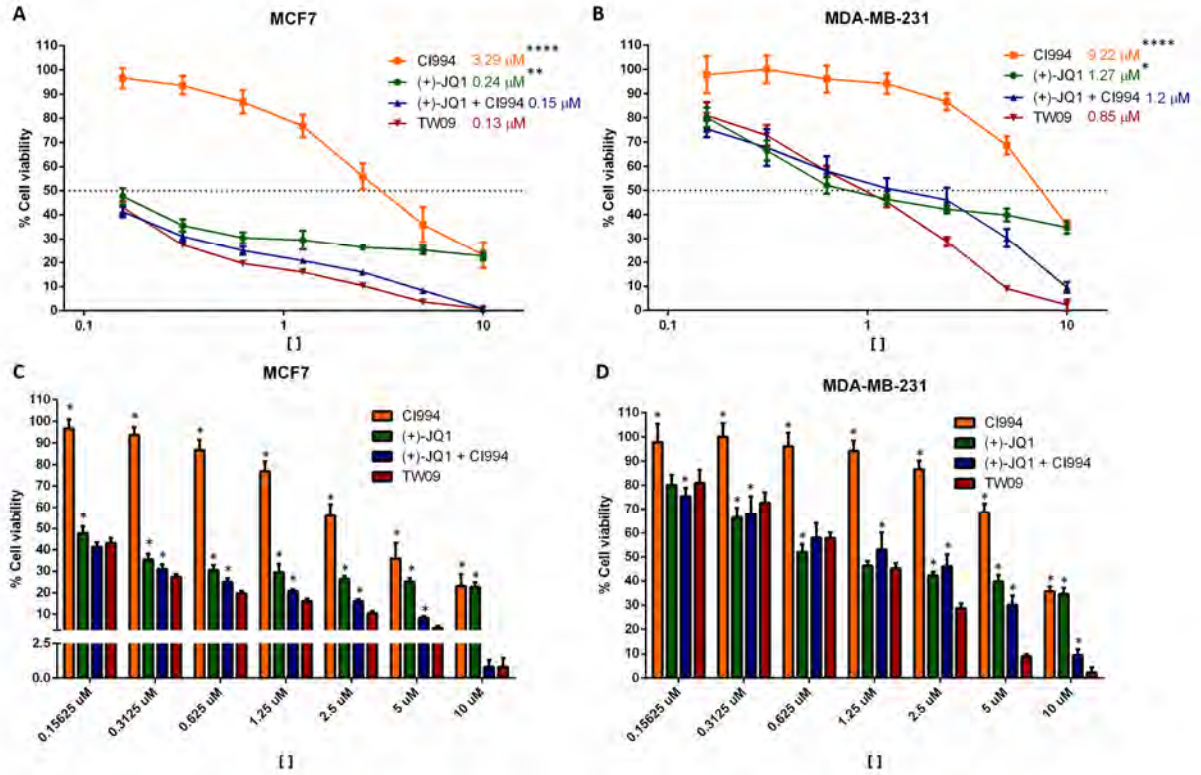


Figure 3. Cell viability of BC cell lines and IC_{50} of CI994, (+)-JQ1, (+)JQ1 + CI994, and TW09 treatment. TW09 IC_{50} was more potent compared to (+)-JQ1 and CI994 in both BC cells (**A-B**). Although did not exhibit a significant lower IC_{50} in comparison with (+)-JQ1 + CI994 co-treatment, TW09 resulted in stronger reduction of cell viability in the most of doses tested in MCF7 cells (**C**) and in the highest doses tested in MDA-MB-231 cells (**D**) (*$p < 0.05$; **$p < 0.01$; **** $p < 0.0001$ – Unpaired T-test was used to compare the IC_{50} between TW09 and other treatments. Two-way ANOVA was used to compare cell viability in different doses tested of TW09 in comparison with (+)-JQ1, CI994, and (+)-JQ1 + CI994 co-treatment).

Autophagy is a process characterized by sequestration of cellular organelles and proteins into acidic vesicles and fusion of these vesicles with lysosomes for degradation. Acridine orange is a lysosomotropic agent able to accumulates in acidic vesicles and emit bright red fluorescence, making possible to track the formation of acidic vesicles and, consequently, autophagy (MURUGAN; AMARAVADI, 2016). Thus, we observed the number of acidic vesicles-positive cells to indicate if treatment with CI994, (+)-JQ1, (+)-JQ1 + CI994 and TW09 in BC cells could induce autophagy. We noticed that all inhibitors could induce accumulation of acidic vesicles in BC cells and this was more eminent in MCF7 cells, confirming the higher susceptibility of these cells to BET and HDAC inhibitors (**Supplementary Figure S3, Appendix D**). Interestingly, dual BET/HDAC inhibition by TW09 increased the number of acidic vesicles-positive cells in both BC cell lines (**Figure 4G and 5G**; **Supplementary Figure 3 and 4, Appendix D**; $p < 0.05$),

suggesting a possible mechanism by which BET/HDAC inhibition can induce cell death in BC cells.

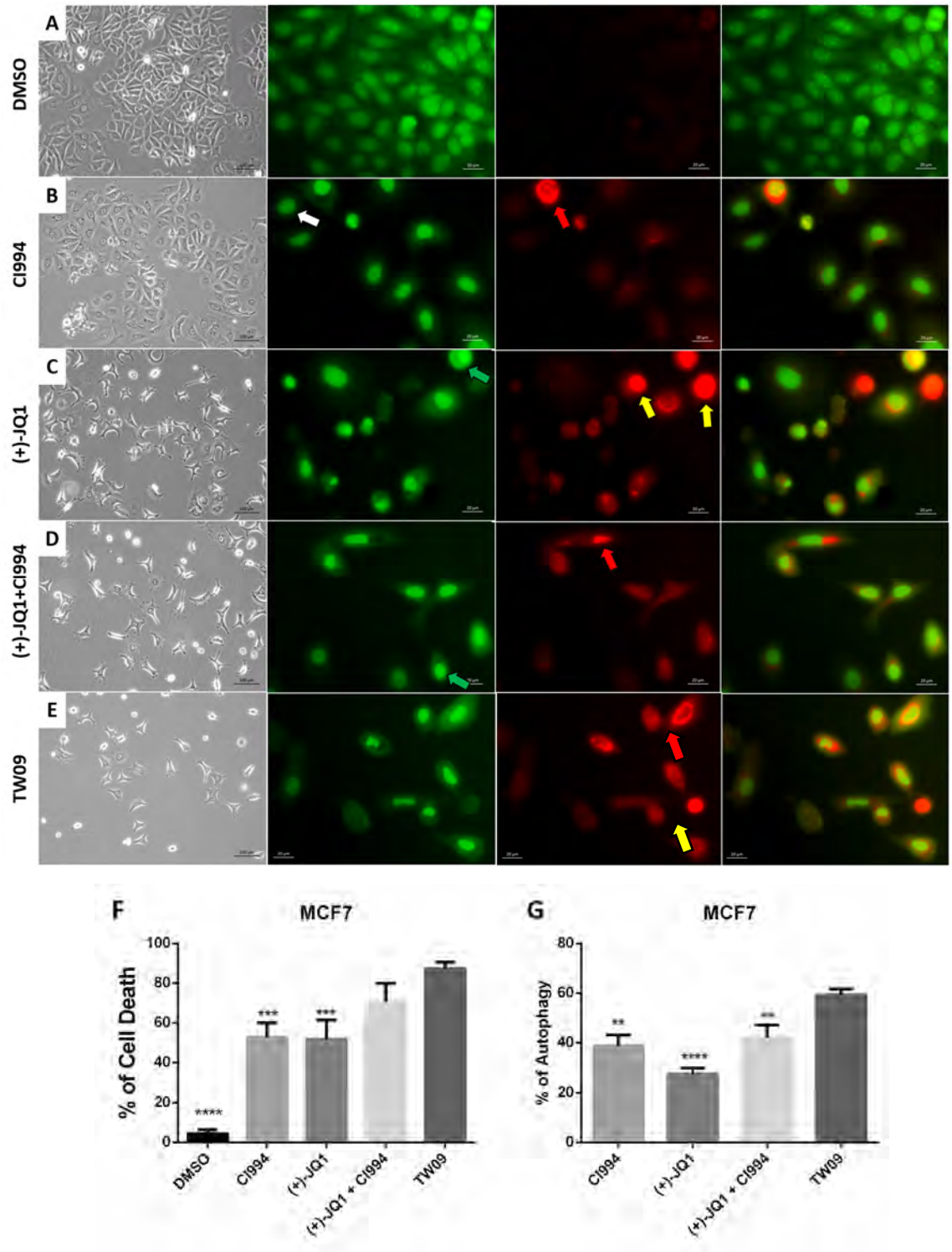


Figure 4. Evaluation of cell death in MCF7 cells treated with CI994, (+)-JQ1, (+)-JQ1 + CI994, and TW09. All inhibitors tested in 2.5 µM induced cell death in MCF7 cells in comparison with the control (DMSO) (**A**). However, dual BET/HDAC inhibition induced by (+)-JQ1+ CI994 co-treatment (**D**) and TW09 (**E**) showed higher cell death in MCF7 cells (**F**). Cell death can be visualized as early apoptosis (green arrow), late

apoptosis (yellow arrow), and oncosis (red arrow). Authopagy, determined by acidic vesicles accumulation stained by AO, was more prevalent in TW09-treated MCF7 cells (**G**) (**$p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$ – One-way ANOVA followed by Dunnett multiple comparison test using TW09 as reference).

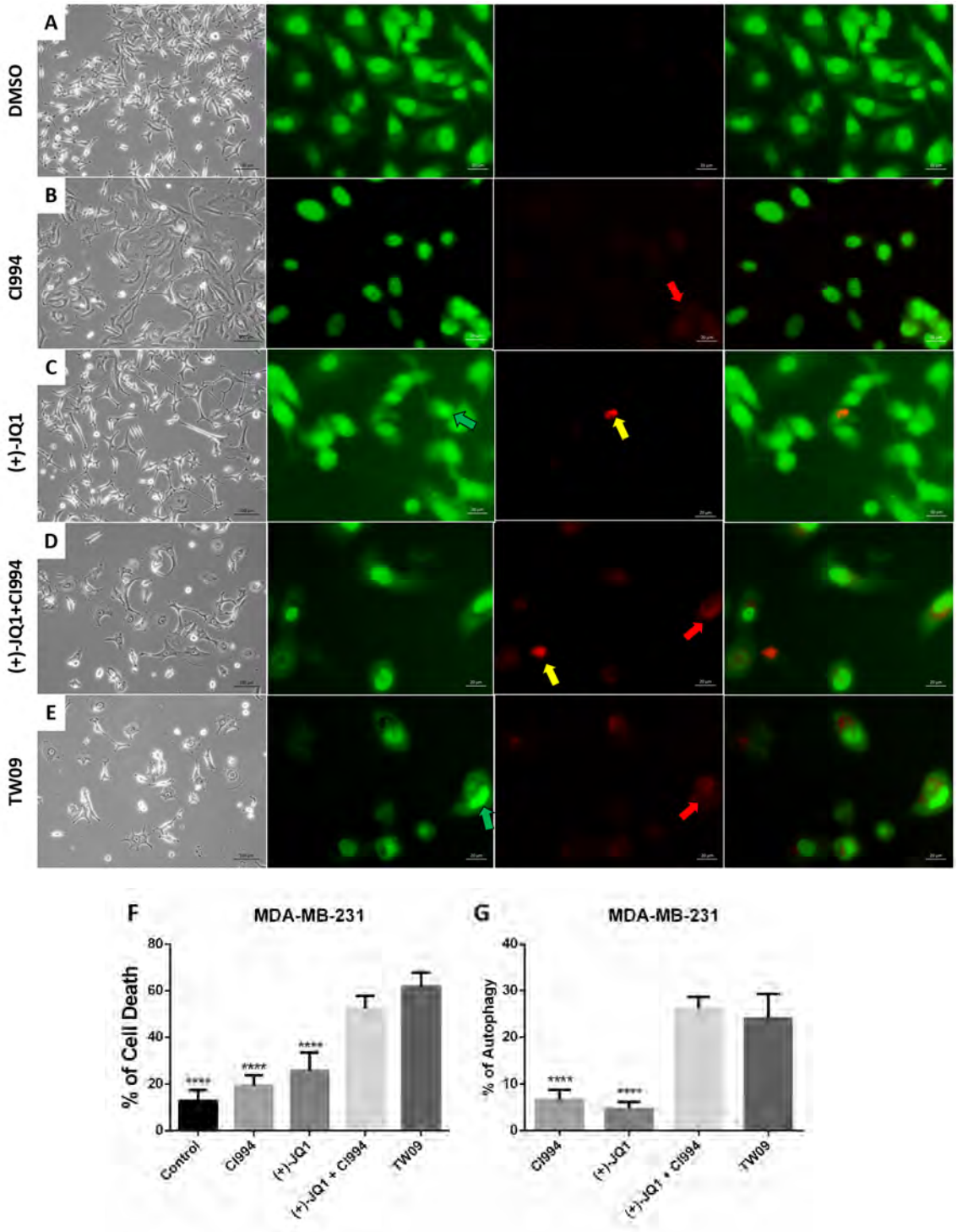


Figure 5. Evaluation of cell death in MDA-MB-231 cells treated with CI994, (+)-JQ1, (+)-JQ1 + CI994, and TW09. All treatments were tested using a concentration of 2.5 µM. Dual BET/HDAC inhibition induced by (+)-JQ1+ CI994 co-treatment (**D**) and TW09 (**E**) induced higher cell death (**F**) in MDA-MB-231 cells in comparison with control (DMSO) (**A**), CI994 (**B**), and (+)-JQ1 (**C**). Cell death can be visualized as early

apoptosis (green arrow), late apoptosis (yellow arrow), and oncosis (red arrow). Autophagy, determined by acidic vesicles accumulation stained by AO, was more prevalent in both (+)-JQ1+CI994 co-treatment and TW09-treated MDA-MB-231 cells (**G**) (**** $p < 0.0001$ – One-way ANOVA followed by Dunnett multiple comparison test using TW09 as reference).

In summary, these results showed that dual BET/HDAC inhibition by TW09 reduces cell viability in a dose-dependent manner on both MCF7 and MDA-MB-231 cell lines and is more efficient than individual BET and HDACs inhibition by (+)-JQ1 and CI994. Furthermore, TW09 is more effective than co-treatment with (+)-JQ1 and CI994 in the most of doses in MCF7 cells and in the highest doses in MDA-MB-231 cells, highlighting TW09 as a potential and efficient strategy to treat HR-positive and TNBC cells, the most malignant and resistant type of BC lacking specific treatment strategies.

5.3.4 TW09 effects on *c-Myc* expression

Targeting BET proteins was demonstrated to inhibit *c-Myc* transcription in many types of cancer (DELMORE et al., 2011). In order to assess if TW09 treatment could downregulate the expression of *c-Myc* in the breast cancer cells, we treated them with 0.5 μM of TW09 for 6h. Then, qPCR was performed to measure *c-Myc* levels. Surprisingly, the levels of *c-Myc* mRNA expression were not significantly decreased after TW09 treatment (**Figure 3**; $p < 0.01$).

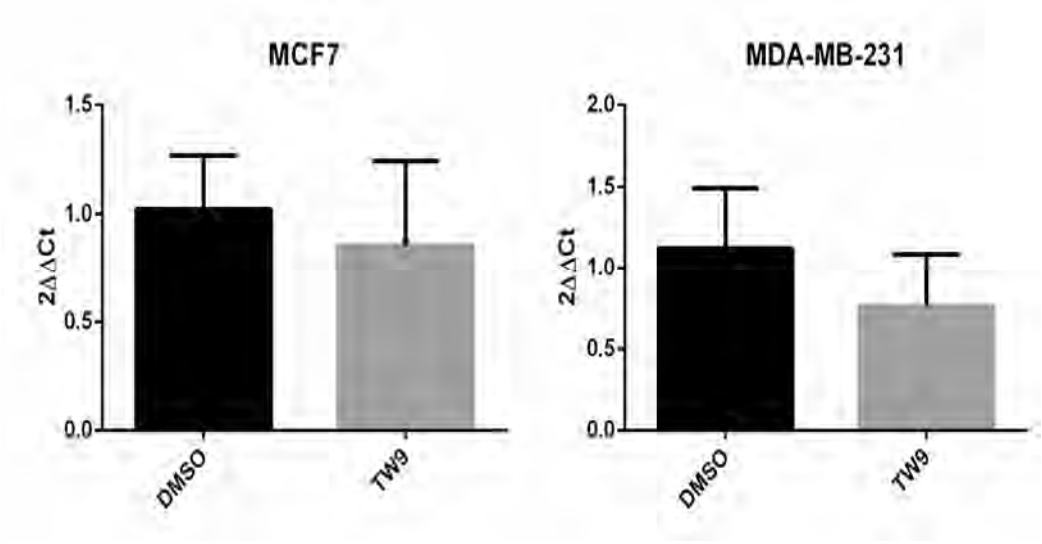


Figure 6. Gene expression analysis of *c-Myc* in TW09-treated and non-treated cells. The *C-myc* expression was not affected in MCF7 and MDA-MB-231 cells after TW09 treatment.

5.3.5 Assessment of TW09 effects on BC tumorspheres

In this project, only the MCF7 cells were able to generate tumorspheres properly (**Supplementary Figure S5**). Then, MCF7 primary tumospheres were treated with TW09 ranging from 0.1 μM to 0.5 μM. All TW09 concentrations decreased the number of tumorspheres and modified the morphological pattern of MCF7 tumorspheres (**Figure 7A-E**; p <0.0001). In addition, although less potent than TW09 in the cell viability assay, low doses of (+)-JQ1 (0.1 μM and 0.5 μM) and CI994 (0.5 μM) also decreased the number of primary MCF7 tumorspheres and modified their shape (**Figure 8**; p < 0.0001).

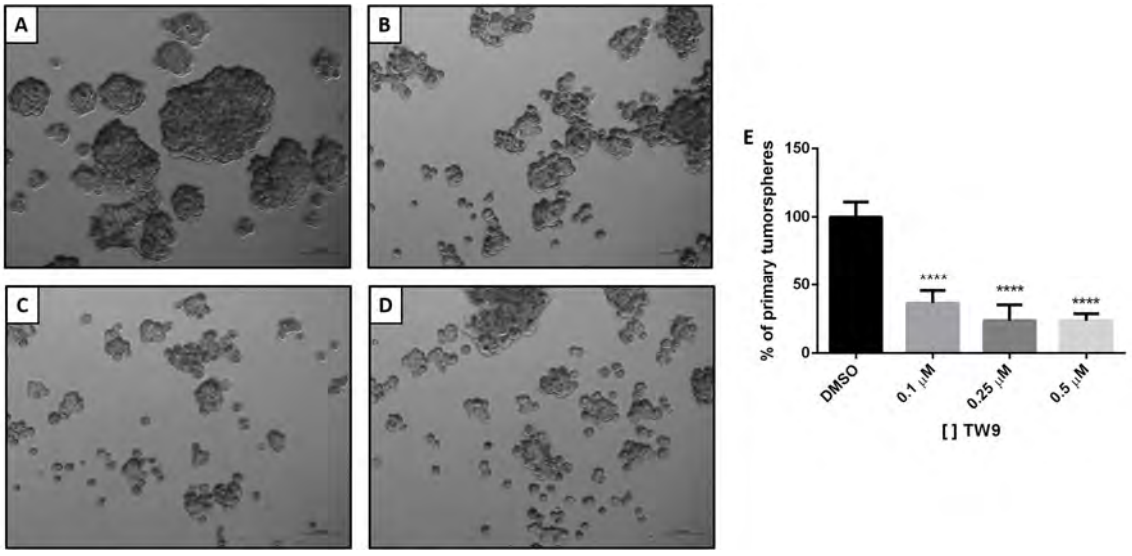


Figure 7. TW09 effects in primary MCF7 tumorspheres. TW09 used at concentrations of 0.1 μM (**B**), 0.25 μM (**C**) and, 0.5 μM (**D**) was able to decrease the number of primary MCF7 tumorspheres (**E**) in comparison with the control (**A**). The magnification of the images is 100X. (**** p < 0.0001 – One-way ANOVA followed by Dunnett multiple comparison test).

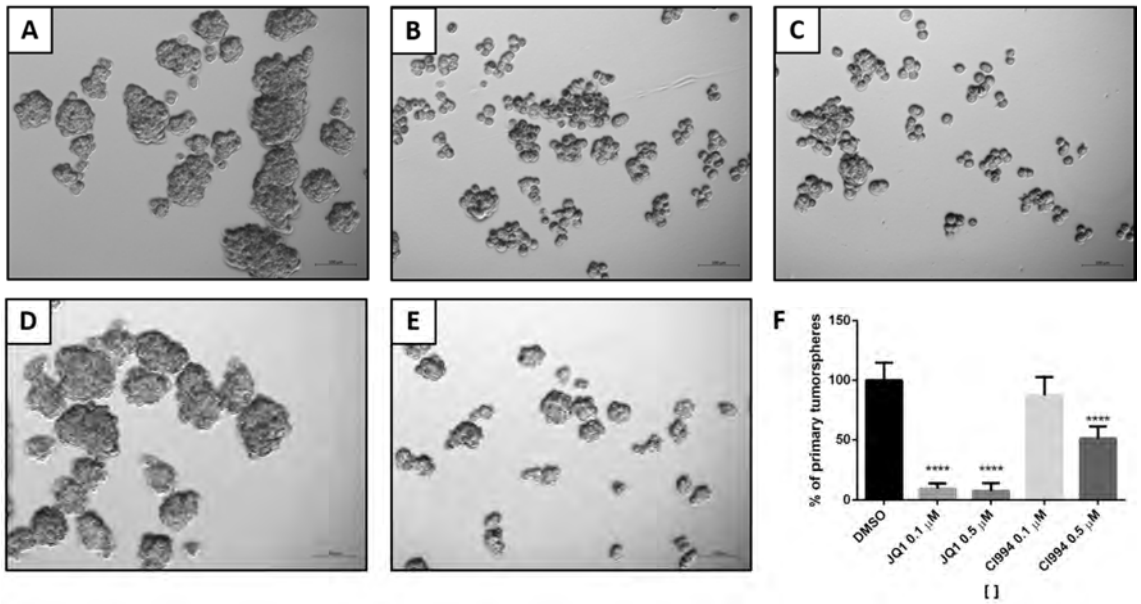


Figure 8. (+)-JQ1 and CI994 effects in primary MCF7 tumorspheres. (+)-JQ1 used at the concentrations of 0.1 μM (**B**) and 0.5 μM (**C**) were able to decrease the number of primary MCF7 tumorspheres (**F**) in comparison with the control (**A**). Only CI994 at the concentration of 0.5 μM was able to decrease the number of primary tumorspheres (**E, F**) while no difference was observed between the control (**A**) and tumorspheres treated with 0.1 μM of CI994 (**D**). The magnification of the images is 100X. (**** $p < 0.001$ – One-way ANOVA followed by Dunnett multiple comparison test).

All TW09 concentrations also significantly inhibited the formation of secondary MCF7 tumorspheres (**Figure 9A-E**; $p < 0.0001$). In addition, TW09 was able to inhibit the formation of secondary tumorspheres, even after removal of the treatment during dissociation of primary tumorspheres, pointing to an epigenetic effect (**Figure 9F-J**; $p < 0.0001$). Finally, we also observed the effects of TW09 in established tumorspheres, after 2 days of growth. In this case, however, concentrations higher than 1 μM of TW09 were necessary to decrease the number of tumorspheres (**Figure 10E;** $p < 0.01$) while they were resistant to low concentrations including 0.1 μM, 0.25 μM, and 0.5 μM (**Figure 10B-D**).

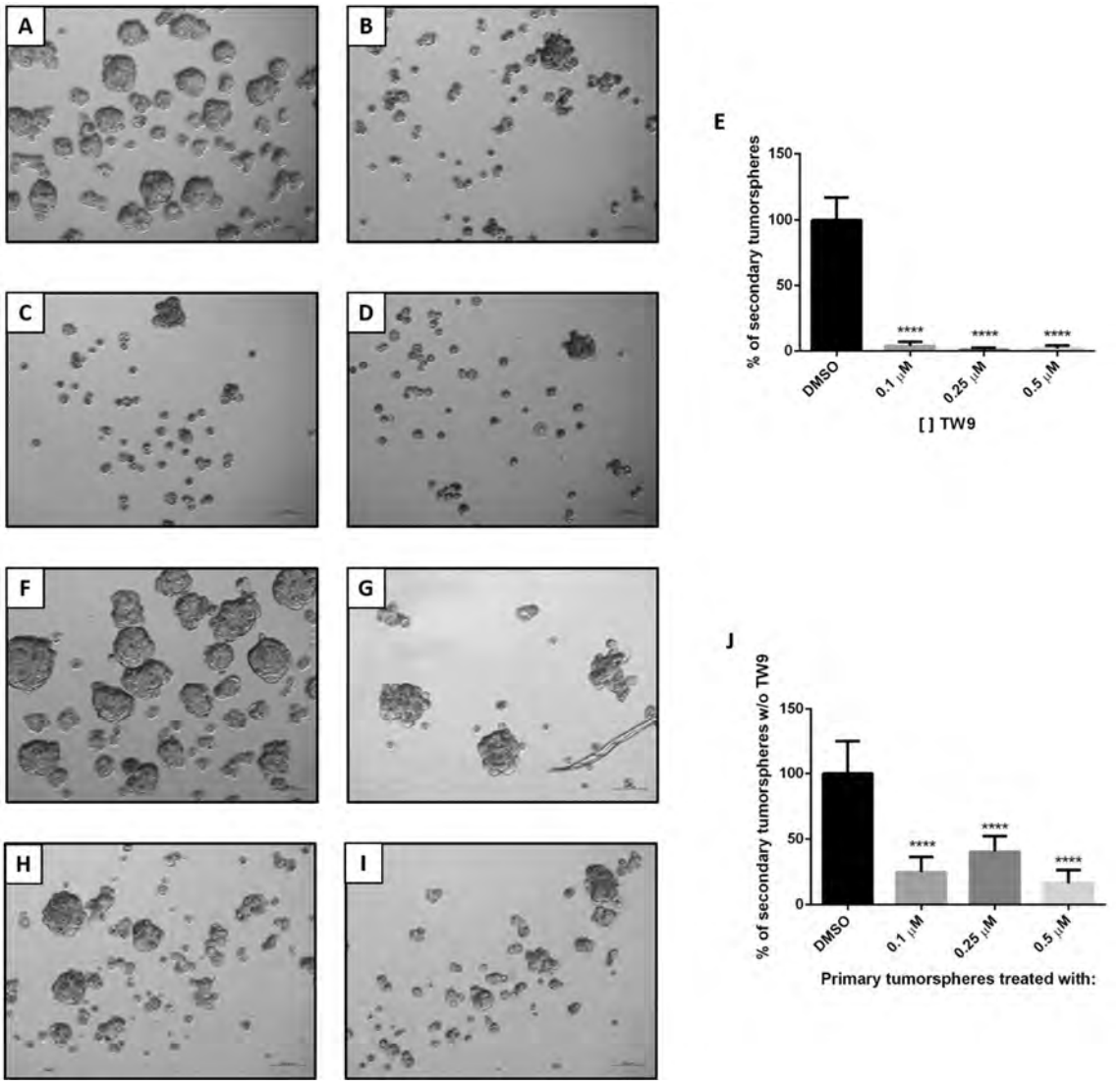


Figure 9. TW09 effects in secondary MCF7 tumorspheres. TW09 used at the concentrations of 0.1 μM (**B**), 0.25 μM (**C**) and, 0.5 μM (**D**) was able to decrease the number of secondary MCF7 tumorspheres (**E**) in comparison with the control (**A**). Furthermore, secondary tumorspheres were formed in smaller numbers (**J**) even after removal of the TW09 treatment with 0.1 μM (**G**), 0.25 μM (**H**) and 0.5 μM (**I**) during dissociation of primary tumorspheres, pointing to an epigenetic effect. The magnification of the images is 100X. (**** $p < 0.001$ – One-way ANOVA followed by Dunnett multiple comparison test).

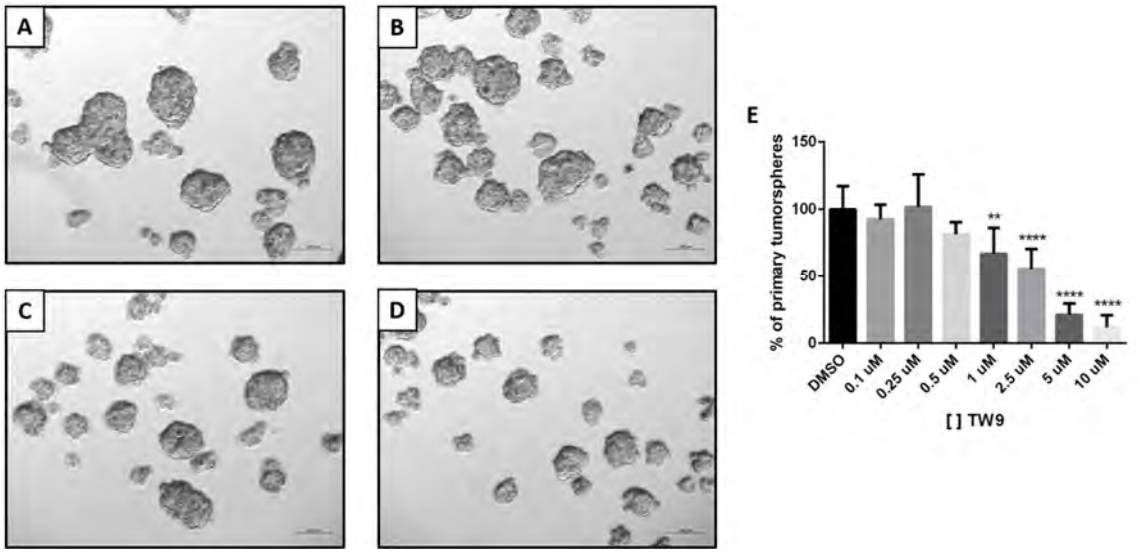


Figure 10. **TW09 effects in established tumorspheres after 2 days of growth**. In this condition, only concentrations ≥ 1 µM were able to decrease the number of tumorspheres (**E**). Low concentrations including 0.1 µM (**B**), 0.25 µM (**C**), and 0.5 µM (**D**) showed no difference in comparison to the control (**A**). The magnification of the images is 100X. (**$p < 0.01$; ****$p < 0.0001$; – One-way ANOVA followed by Dunnett multiple comparison test).

5.3.6 Cell death pattern in TW09-treated tumorspheres

To assess if TW09 is inducing apoptosis in the tumorspheres we used two different assays: Caspase-3 staining and Annexin V/Yo-pro3 staining. Tumorspheres treated with 0.1 µM, 0.25 µM and 0.5 µM showed only a weak caspase-3 staining in few cells of the tumorspheres (**Figure 11**). A faint annexin V/Yo-pro3 staining could also be observed in tumorspheres treated with 0.1 µM, 0.25 µM and 0.5 µM of TW9 (**Figure 12**). However, in established tumorspheres, annexin V/Yo-pro3 staining was only observed at concentrations higher than 2.5 µM of TW09 treatment (**Figure 13**). The results described here suggest that low concentrations of TW09 as 0.1 µM, 0.25 µM, and 0.5 µM inhibited the formation of tumorspheres by mechanisms not associated with apoptosis as evidenced by low staining of caspase-3 and Annexin V/Yo-pro3 in the tumorspheres treated at these concentrations. On the other hand, higher concentrations of TW09 induce apoptosis in the tumorspheres.

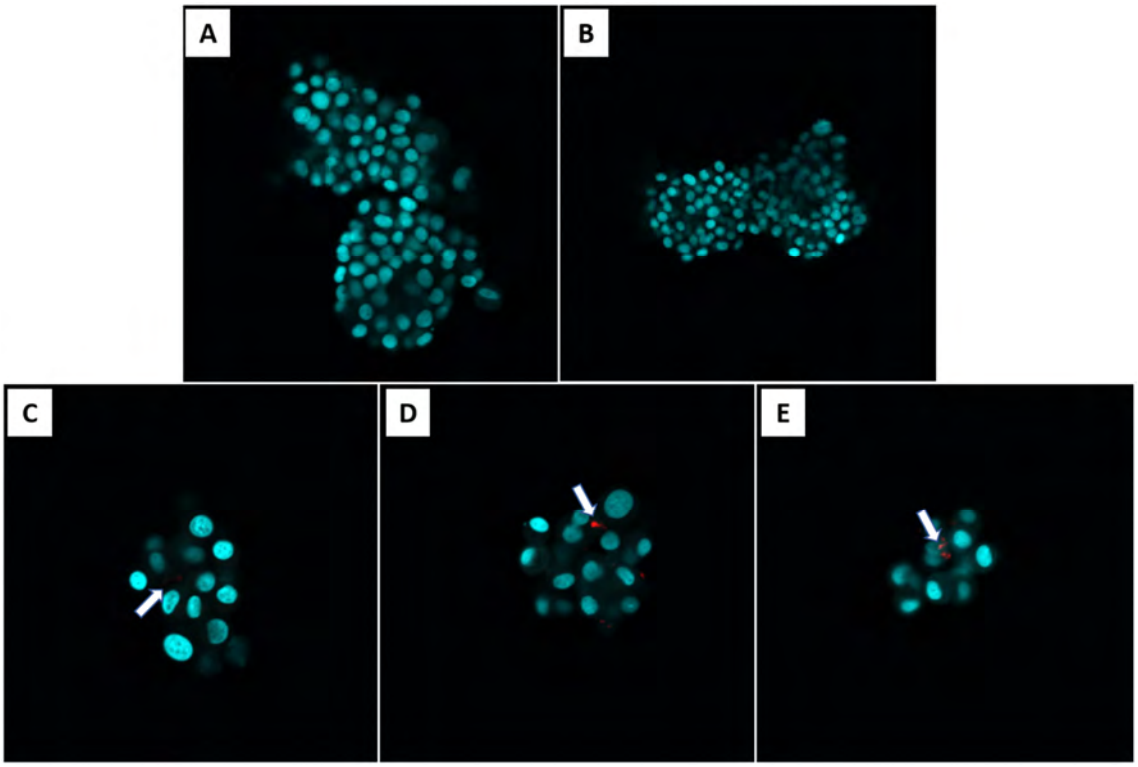


Figure 11. Caspase-3 staining (Red) of TW09-treated tumorspheres. Treatment with 0.1 µM (**C**), 0.25 µM (**D**) and, 0.5 µM (**E**) slightly induced the expression of caspase-3 in the tumorspheres. It was not possible to observe this staining in control tumorspheres (DMSO) (**A**) and tumospheres without the presence of the primary antibody (**B**) excluding the possibility of false positives. Cell nuclei are labeled with Hoechst 33342 (Blue).

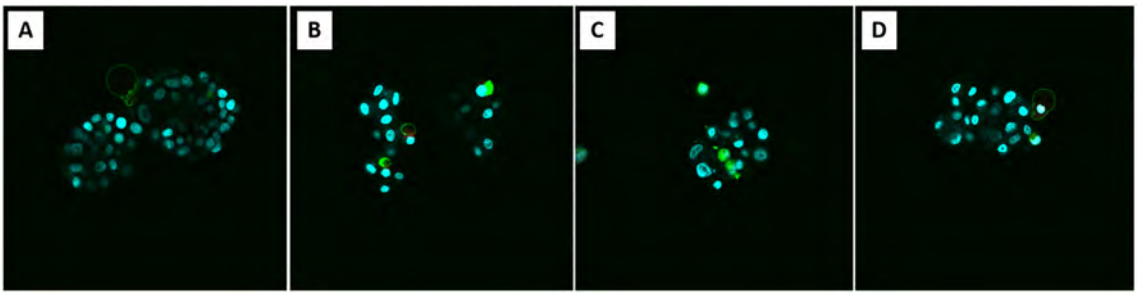


Figure 12. Annexin V/Yo-pro3 staining of TW09-treated tumorspheres. We could observe staining of Annexin V/yo-pro3 only in few cells inside the tumorspheres treated with 0.1 µM (**B**), 0.25 µM (**C**), and 0.5 µM (**D**). Cell nuclei are labeled with Hoechst 33342 (Blue). Annexin V is labeled in green and Yo-pro3 in red.

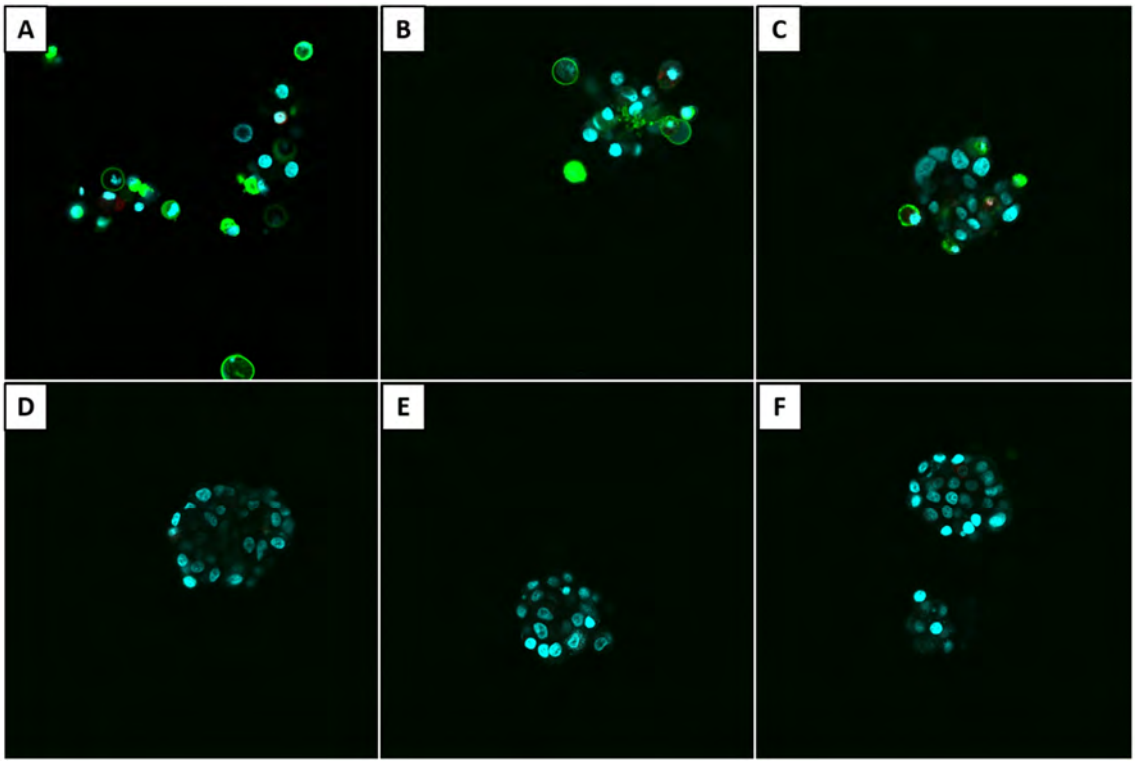


Figure 13. Annexin V/Yo-pro3 staining in establish tumorspheres treated with TW09 after 2 days of growth. Annexin V/Yo-pro3 staining was only observed at higher concentrations of TW09 including 10 µM (**A**), 5 µM (**B**), and 2.5 µM (**C**). We did not observe staining in tumorspheres treated with lower concentrations of TW09 including 0.5 µM (**D**), 0.25 µM (**E**) and, 0.1 µM (**F**). Cell nuclei are labeled with Hoechst 33342 (Blue). Annexin V is labeled in green and Yo-pro3 in red.

5.3.7 Effects of TW09 in Canine Mammary Cancer cells

We also established the first steps to test TW09 in canine mammary cancer cells, to compare the effects of TW09 in both human and canine species. So far, TW09 induced cell viability reduction in a dose-dependent manner in CF41. Mg cells and a significant lower IC_{50} ($p < 0.05$) in comparison with CI994, but no with (+)-JQ1.

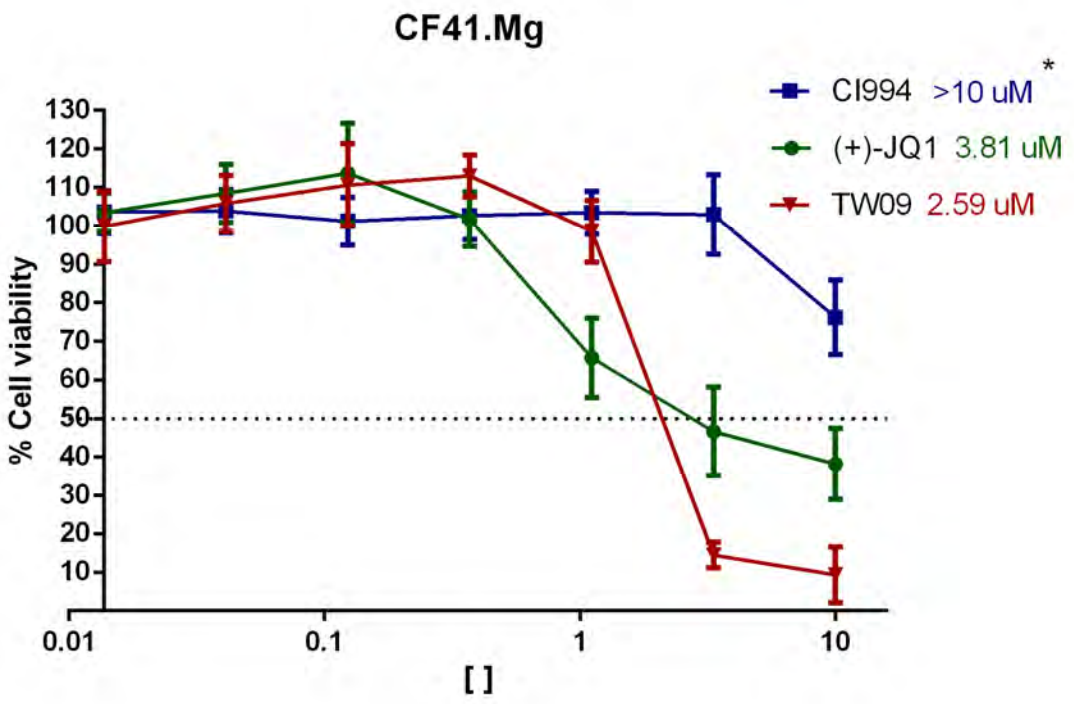


Figure 14. Cell viability of CF41.Mg cell lines and IC_{50} of CI994, (+)-JQ1, and TW09 treatment. TW09 reduced cell viability in a dose dependent manner in CF41.Mg cells and was more potent compared to CI994 but no with (+)-JQ1. (*$p < 0.05$ – Unpaired T-test was used to compare the IC_{50} between TW09 and other treatments).

5.4 Discussion

In the present study, we report the effects of dual HDAC-BET inhibition by a new inhibitor, TW09, on cell viability, cell death, gene expression of *c-Myc*, and tumorspheres formation in two different types of breast cancer cell lines: HR-positive breast cancer and TNBC. TW09 decreased the cell viability and increased cell death of both HR-positive and TNBC cell lines and was more potent in comparison to the individual inhibitors (+)-JQ1 (BET inhibitor) and CI994 (HDAC inhibitor). Furthermore, HDAC-BET inhibition by TW09 decreased the number of BC tumorspheres suggesting a promising strategy to target tumorigenicity and self-renewal in breast cancer.

Both HDAC and BET epigenetic proteins have been shown to play key roles in cancer and are considered attractive therapeutic targets (MANZOTTI; CIARROCCHI; SANCISI, 2019). We confirmed the high expression of BRD4 and HDAC in all the breast cancer cells such as previous studies (TANG et al., 2017; LU et al., 2020). Then, we notice that BET/HDAC inhibition decreased cell viability of breast cancer cell lines,

demonstrating a promising strategy to treat breast cancer cells likewise has been shown in other types of cancer including to lymphoma (BHADURY et al., 2014), leukemia (FISKUS et al., 2014), pancreatic adenocarcinoma (MAZUR et al., 2015), melanoma (HEINEMANN et al., 2015) and glioblastoma (ZHANG et al., 2018). Specifically, BET/HDAC inhibition by TW09 has recently shown potent antitumor activity in rhabdomyosarcoma cells, a type of soft-tissue sarcoma (LASZIG et al., 2020). Furthermore, the approach used in this study, using a dual-targeting agent, may overcome some issues observed when applying individual targeting agents including incomplete efficacy and drug resistance which may result in a rational strategy against cancer (STANKOVIĆ et al., 2018).

Triple-negative breast cancer (TNBC) is considered the most malignant type of breast cancer and defined by the lack of estrogen receptor (ER), progesterone receptor (PR) and, HER2 expression. In addition to being associated with poor patient prognosis of patients, is a type of breast cancer lacking specific treatment strategies and patients frequently develop resistance to chemotherapy (BIANCHINI et al., 2016; NEDELJKOVIĆ; DAMJANOVIĆ, 2019). We showed that TNBC cell line, MDA-MB-231, were more resistant to individual inhibitors of BET proteins ((+)-JQ1), and HDACs (CI994). However, dual HDAC-BET inhibition using (+)-JQ1 + CI994 co-treatment and TW09 was stronger to decrease cell viability and induce cell death in these cells. Similar results were showed when MDA-MB-231 cells were treated with (+)-JQ1 and Mocetinostat, another HDAC inhibitor. This combination presented a synergistic effect reducing cell viability of TNBC cells (BORBELY et al., 2015). Thus, these results can suggest and highlight dual HDAC-BET inhibition as an efficient and promising strategy to treat triple-negative breast cancer.

BET and HDAC inhibition has previously been described to induce autophagy in different types of cancer (SHAO et al., 2004; LI et al., 2019). In our results, we showed that dual BET/HDAC inhibition by (+)-JQ1 + CI994 co-treatment and TW09 increased the number of cells in autophagic process observed by acidic vesicles accumulation. These results could suggest that autophagy was one of mechanisms inducing higher cell death activity in BC cells after dual BET/HDAC inhibition. However, at the same time, we also could observe autophagy signals, even if in a smaller proportion, in BC cells treated with (+)-JQ1 and CI994 individually, which induced lower cell death rates.

Autophagy is a cellular mechanism to recycling and cellular self-degradation important to maintain metabolism and homeostasis. Substantial evidences has been proposed autophagy as a double-edged sword in cancer, promoting both tumor-suppressive or tumor-promoting activity. Tumor cells can activate autophagy in response to prolonged stress "eating" themselves, suppressing cell division and motility and being important to conserve energy, maintaining viability and facilitating recovery (WHITE; DIPAOLA, 2009). In addition, a series of studies observed that autophagy can confers resistance to cancer therapy, including resistance to BET inhibition by (+)-JQ1 and HDAC inhibitors (JANG et al., 2016; MRAKOVCIC; FRÖHLICH, 2020). In contrast, autophagy has also been observed as a type of cell death in cancer cells in prolonged stress and progressive autophagy, inducing damage amplification and senescence and some studies demonstrated BET and HDAC inhibition inducing cell death in cancer cells (SHAO et al., 2004; LI et al., 2019). Thus, only the results showed here, prevent us to confirm that autophagy is the mechanism responsible to induce cell death in BC cells treated with (+)-JQ1 + CI994 and TW09. Therefore, more studies are necessary to determine the function of autophagy in BC cells treated with BET/HDAC inhibitors.

BET proteins are considered a regulatory factor of *c-Myc* and targeting BET proteins was discovered to inhibit *c-Myc* transcription in many types of cancer (DELMORE et al., 2011; TOGEL et al., 2016). Here, treatment with TW09 not affected levels of *c-Myc* mRNA expression in BC cells. In human breast cancer, BRD4 plays an important role in breast tumor proliferation (NAGARAJAN et al., 2014) and BET inhibition has been shown to contribute to overcoming resistance in HER2 and hormone receptors positive tumors (HR) (FENG et al., 2014; STUHLMILLER et al., 2015). However, triple-negative breast cancer (TNBC), the most aggressive subtype, is not commonly associated with BRD4/MYC regulation (SAHNI et al., 2016; SHU et al., 2016). In addition, our recent study also has shown that (+)-JQ1 treatment was not able to decrease the *c-Myc* expression in canine mammary cancer cells (XAVIER et al., 2019).

The use of three-dimensional *in vitro* models (3D) using tumorspheres has been widely used to study cancer phenotypes including tumorigenicity and self-renewal (WEISWALD; BELLET; DANGLES-MARIE, 2015). Thus, the effects of TW09 decreasing the number of primary and secondary tumorspheres suggest a potential strategy to inhibit these phenotypes in breast cancer. Previous studies have demonstrated

individual BET and HDAC inhibition as an approach to avoid these phenotypes (VENKATARAMAN et al., 2014; PATHANIA et al., 2016; XAVIER et al., 2019). However, there is no study observing the effects of combined HDAC-BET inhibition on self-renewal and tumorigenicity of breast cancer. In addition, we observed that concentrations of TW09 as 0.1 μM, 0.25 μM, and 0.5 μM inhibited the formation of tumorspheres by mechanisms not associated with apoptosis as evidenced by low staining of caspase-3 and Annexin V/Yo-pro3 in the tumorspheres treated at these concentrations. Recently, we observed similar results when we used low concentrations of (+)-JQ1 (BET inhibitor) to inhibit self-renewal and tumorigenicity in canine mammary cancer cells (XAVIER et al., 2019), suggesting that our findings may begin to elucidate combined inhibition of BET and HDAC proteins as promising in restraining these phenotypes in mammary cancer.

5.5 Conclusion

Our findings showed that dual BET-HDAC inhibition by TW09 shows positive effects to treat both HR-positive and TNBC cells, decreasing the cell viability and number of tumorspheres, suggesting effects regarding tumorigenicity and self-renewal potential of breast cancer cells. Thus, may provide a promising treatment against breast cancer, in particular for the more resistant triple-negative cells. Now, we aim to elucidate possible mechanisms by which TW09 is inducing cell death and blocking some phenotypes of breast cancer cells. Finally, we will seek to observe the same effects of the TW09 in canine mammary cancer cells, providing a comparative study between breast cancer and canine mammary cancer.

5.6 References

ALEKSEYENKO, A. A. et al. Ectopic protein interactions within BRD4–chromatin complexes drive oncogenic megadomain formation in NUT midline carcinoma. **Proceedings of the National Academy of Sciences**, v. 114, n. 21, p. E4184–E4192, 2017.

BHADURY, J. et al. BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. **Proceedings of the National Academy of Sciences of the United States of America**, v. 111, n. 26, p. 2721–2730, 2014.

BIANCHINI, G. et al. Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. **Nature Reviews Clinical Oncology**, v. 13, n. 11, p. 674–690, 2016.

BORBELY, G. et al. Induction of USP17 by combining BET and HDAC inhibitors in breast cancer cells. **Oncotarget**, v. 6, n. 32, p. 33623–33635, 2015.

BUTLER, L. M. et al. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 18, p. 11700–11705, 2002.

CHOU, T. C.; TALALAY, P. Analysis of combined drug effects: a new look at a very old problem. **Trends in Pharmacological Sciences**, v. 4, n. C, p. 450–454, 1983.

CLAYTON, A. L.; HAZZALIN, C. A.; MAHADEVAN, L. C. Enhanced Histone Acetylation and Transcription: A Dynamic Perspective. **Molecular Cell**, v. 23, n. 3, p. 289–296, 2006.

DAWSON, M. A.; KOUZARIDES, T. Cancer epigenetics: From mechanism to therapy. **Cell**, v. 150, n. 1, p. 12–27, 2012. Disponível em: <http://dx.doi.org/10.1016/j.cell.2012.06.013>.

DELMORE, J. E. et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. **Cell**, v. 146, n. 6, p. 904–917, 2011. Disponível em: <http://dx.doi.org/10.1016/j.cell.2011.08.017>.

FENG, Q. et al. An epigenomic approach to therapy for tamoxifen-resistant breast cancer. **Cell Research**, v. 24, n. 7, p. 809–819, 2014. Disponível em: <http://dx.doi.org/10.1038/cr.2014.71>.

FILIPPAKOPOULOS, P. et al. Selective inhibition of BET bromodomains. **Nature**, v. 468, n. 7327, p. 1067–1073, 2010.

FISKUS, W. et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. **Molecular Cancer Therapeutics**, v. 13, n. 5, p. 1142–1154, 2014.

GALANIS, E. et al. Phase II trial of Vorinostat in recurrent glioblastoma multiforme: A north central cancer treatment group study. **Journal of Clinical Oncology**, v. 27, n. 12, p. 2052–2058, 2009.

GLOZAK, M. A.; SETO, E. Histone deacetylases and cancer. **Oncogene**, v. 26, n. 37, p. 5420–5432, 2007.

GUO, S. Q.; ZHANG, Y. Z. Histone deacetylase inhibition: an important mechanism in the treatment of lymphoma. **Cancer Biology and Medicine**, v. 9, n. 2, p. 85–89, 2012.

HABERLAND, M.; MONTGOMERY, R. L.; OLSON, E. N. Physiology : Implications for Disease and Therapy. **Nature Reviews. Genetics**, v. 10, n. 1, p. 32–42, 2011.

HEINEMANN, A. et al. Combining BET and HDAC inhibitors synergistically induces apoptosis of melanoma and suppresses AKT and YAP signaling. **Oncotarget**, v. 6, n.

25, p. 21507–21521, 2015.

HÖLSCHER, A. S. et al. Combined inhibition of BET proteins and class I HDACs synergistically induces apoptosis in urothelial carcinoma cell lines. **Clinical Epigenetics**, v. 10, n. 1, p. 1–14, 2018.

IANEVSKI, A. et al. SynergyFinder: A web application for analyzing drug combination dose-response matrix data. **Bioinformatics**, v. 33, n. 15, p. 2413–2415, 2017.

JANG, J. E. et al. AMPK-ULK1-mediated autophagy confers resistance to BET inhibitor JQ1 in acute myeloid leukemia stem cells. **Clinical Cancer Research**, v. 23, n. 11, p. 2781–2794, 2017.

JONES, P. A.; BAYLIN, S. B. The Epigenomics of Cancer. **Cell**, v. 128, n. 4, p. 683–692, fev. 2007. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0092867407001274>.

JOSLING, G. A. et al. The role of bromodomain proteins in regulating gene expression. **Genes**, v. 3, n. 2, p. 320–343, 2012.

KOMATSU, N. et al. SAHA, a HDAC inhibitor, has profound anti-growth activity against non-small cell lung cancer cells. **Oncology Reports**, v. 15, n. 1, p. 187–191, 2006.

LASZIG, S. et al. The novel dual BET/HDAC inhibitor TW09 mediates cell death by mitochondrial apoptosis in rhabdomyosarcoma cells. **Cancer Letters**, v. 486, n. April, p. 46–57, 2020. Disponível em: <https://doi.org/10.1016/j.canlet.2020.05.008>.

LI, Z.; ZHU, W. G. Targeting histone deacetylases for cancer therapy: From molecular mechanisms to clinical implications. **International Journal of Biological Sciences**, v. 10, n. 7, p. 757–770, 2014.

LI, F. et al. BET inhibitor JQ1 suppresses cell proliferation via inducing autophagy and activating LKB1/AMPK in bladder cancer cells. **Cancer Medicine**, v. 8, n. 10, p. 4792–4805, 2019.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. **Methods**, v. 25, n. 4, p. 402–408, 2001.

LU, L. et al. Inhibition of BRD4 suppresses the malignancy of breast cancer cells via regulation of Snail. **Cell Death and Differentiation**, v. 27, n. 1, p. 255–268, 2020. Disponível em: <http://dx.doi.org/10.1038/s41418-019-0353-2>.

MANZOTTI, G.; CIARROCCHI, A.; SANCISI, V. Inhibition of BET proteins and histone deacetylase (HDACs): Crossing roads in cancer therapy. **Cancers**, v. 11, n. 3, 2019.

MARTIN, N. et al. CompuSyn by Ting-Chao Chou. v. 2005, n. D, p. 3–4, 2010.

MAZUR, P. K. et al. Combined inhibition of BET family proteins and histone deacetylases as a potential epigenetics-based therapy for pancreatic ductal adenocarcinoma. **Nature Medicine**, v. 21, n. 10, p. 1163–1171, 2015.

MERTZ, J. A. et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. **Proceedings of the National Academy of Sciences**, v. 108, n. 40, p. 16669–16674, out. 2011.

MONTENEGRO, R. C. et al. BET inhibition as a new strategy for the treatment of gastric cancer. **Oncotarget**, v. 7, n. 28, p. 43997–44012, 2016.

MRAKOVCIC, M.; FRÖHLICH, L. F. Molecular determinants of cancer therapy resistance to HDAC inhibitor-induced autophagy. **Cancers**, v. 12, n. 1, p. 1–30, 2020.

MULLER, S.; FILIPPAKOPOULOS, P.; KNAPP, S. Bromodomains as therapeutic targets. **Expert Reviews in Molecular Medicine**, v. 13, n. September 2011, p. e29, 2011. Disponível em: <http://www.journals.cambridge.org/abstract_S1462399411001992>.

MURUGAN, S.; AMARAVADI, R. K. Methods for studying autophagy within the tumor microenvironment. **Advances in Experimental Medicine and Biology**, v. 899, p. 145–166, 2016.

NAGARAJAN, S. et al. Bromodomain Protein BRD4 Is Required for Estrogen Receptor-Dependent Enhancer Activation and Gene Transcription. **Cell Reports**, v. 8, n. 2, p. 460–469, 2014.

NEDELJKOVIĆ, M.; DAMJANOVIĆ, A. Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer—How We Can Rise to the Challenge. **Cells**, v. 8, n. 9, p. 957, 2019.

OCAÑA, A.; NIETO-JIMÉNEZ, C.; PANDIELLA, A. BET inhibitors as novel therapeutic agents in breast cancer. **Oncotarget**, v. 8, n. 41, p. 71285–71291, 2017.

PATHANIA, R. et al. Combined inhibition of DNMT and HDAC blocks the tumorigenicity of cancer stem-like cells and attenuates mammary tumor growth. **Cancer Research**, v. 76, n. 11, p. 3224–3235, 2016.

RAINA, K. et al. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. **Proceedings of the National Academy of Sciences of the United States of America**, v. 113, n. 26, p. 7124–7129, 2016.

RICHON, V. M. Cancer biology: Mechanism of antitumour action of vorinostat (suberoylanilide hydroxamic acid), a novel histone deacetylase inhibitor. **British Journal of Cancer**, v. 95, n. SUPPL. 1, p. 2–6, 2006.

RODRIGUEZ, R. M. et al. Aberrant epigenetic regulation of bromodomain Brd4 in

human colon cancer. **Journal of Molecular Medicine**, v. 90, n. 5, p. 587–595, 2012.

SAHNI, J. M. et al. Bromodomain and extraterminal protein inhibition blocks growth of triple-negative breast cancers through the suppression of Aurora kinases. **Journal of Biological Chemistry**, v. 291, n. 45, p. 23756–23768, 2016.

SCHAEFER, E. W. et al. A phase 2 study of vorinostat in acute myeloid leukemia. **Haematologica**, v. 94, n. 10, p. 1375–1382, 2009.

SHAO, Y. et al. Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. **Proceedings of the National Academy of Sciences of the United States of America**, v. 101, n. 52, p. 18030–18035, 2004.

SHU, S. et al. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. **Nature**, v. 529, n. 7586, p. 413–417, 2016. Disponível em: <http://dx.doi.org/10.1038/nature16508>.

STANKOVIĆ, T. et al. Dual Inhibitors as a New Challenge for Cancer Multidrug Resistance Treatment. **Current Medicinal Chemistry**, v. 26, n. 33, p. 6074–6106, 2018.

STUHLMILLER, T. J. et al. Inhibition of lapatinib-induced kinome reprogramming in ERBB2-positive breast cancer by targeting BET family bromodomains. **Cell Reports**, v. 11, n. 3, p. 390–404, 2015. Disponível em: <http://dx.doi.org/10.1016/j.celrep.2015.03.037>.

TANAKA, M. et al. Design and characterization of bivalent BET inhibitors. **Nature Chemical Biology**, v. 12, n. 12, p. 1089–1096, 2016.

TANG, Z. et al. HDAC1 triggers the proliferation and migration of breast cancer cells via upregulation of interleukin-8. **Biological Chemistry**, v. 398, n. 12, p. 1347–1356, 2017.

TOGEL, L. et al. Dual Targeting of Bromodomain and Extraterminal Domain Proteins, and WNT or MAPK Signaling, Inhibits c-MYC Expression and Proliferation of Colorectal Cancer Cells. **Molecular Cancer Therapeutics**, v. 15, n. 6, p. 1217–1226, 2016.

VALLONE, P. M.; BUTLER, J. M. AutoDimer: A screening tool for primer-dimer and hairpin structures. **BioTechniques**, v. 37, n. 2, p. 226–231, 2004.

VENKATARAMAN, S. et al. Inhibition of BRD4 attenuates tumor cell self-renewal and suppresses stem cell signaling in MYC driven medulloblastoma. **Oncotarget**, v. 5, n. 9, p. 2355–71, 2014. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4058011&tool=pmcentrez&rendertype=abstract>.

WEISWALD, L. B.; BELLET, D.; DANGLES-MARIE, V. Spherical Cancer Models in Tumor Biology. **Neoplasia (United States)**, v. 17, n. 1, p. 1–15, 2015. Disponível em:

<http://dx.doi.org/10.1016/j.neo.2014.12.004>.

WHITE, E.; DIPAOLA, R. S. The double-edged sword of autophagy modulation in cancer. **Clinical Cancer Research**, v. 15, n. 17, p. 5308–5316, 2009.

XAVIER, P. L. P. et al. An epigenetic screening determines BET proteins as targets to suppress self-renewal and tumorigenicity in canine mammary cancer cells. **Scientific Reports**, v. 9, n. 1, p. 1–15, 2019.

YANG, X. J.; SETO, E. HATs and HDACs: From structure, function and regulation to novel strategies for therapy and prevention. **Oncogene**, v. 26, n. 37, p. 5310–5318, 2007.

YANG, Z. et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. **Molecular Cell**, v. 19, n. 4, p. 535–545, 2005.

YE, J. et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. **BMC bioinformatics**, v. 13, p. 134, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22708584%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3412702>.

ZHANG, Y. et al. Combined HDAC and bromodomain protein inhibition reprograms tumor cell metabolism and elicits synthetic lethality in glioblastoma. **Clinical Cancer Research**, v. 24, n. 16, p. 3941–3954, 2018.

ZUBER, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. **Nature**, v. 478, n. 7370, p. 524–528, out. 2011.

7. General Conclusion and Perspectives

Throughout this thesis, we presented findings that contributed to the enrichment of a field widely explored in human oncology but still few explored in veterinary oncology: the epigenetics. According to PubMed, 19.005 studies regarding “epigenetics and human cancer” have been published between 2015-2019. In contrast, only 55 studies could be found, in the same period, when we search for “epigenetics and canine cancer”. These numbers demonstrate the focus and efforts on developing human healthy but, at the same time, highlight a wide field that we have to still explore in science. The findings we gathered in the first chapter suggest that many of the well-described epigenetic alterations in different types of human cancer are also observed in canine cancer including classical genome-wide hypomethylation, hypermethylation of tumor supressor genes, aberrant histone modifications and non-coding RNAs, highlighting a potential approach using canine model to determine new epigenetic mechanisms behind cancer, diagnostic/prognostic markers and targets for the development of new anticancer drugs.

In chapter 2, using *in vitro* models, we observed possible genes and signaling pathways regulating important phenotypes to cancer initiation and progression in canine mammary cancer, such as tumorigenicity and invasiveness. The ZEBs transcription factors and TGF-β signaling, for example, are very well-described and important inducing EMT and, consequently, tumorigenicity and metastasis in many different types of human cancer. There are several TGF-β signaling inhibitors such as neutralizing antibodies, ligand traps, and receptor kinase inhibitors that have been tested in (pre)clinical trials in humans such as LY3022859, an anti-TβRII monoclonal antibody that inhibits receptor-mediated TGF-β signaling activation (TOLCHER et al., 2017) and the TGF- β receptor trap, RER (ZHU et al., 2018). In chapter 3, we demonstrated the effects of (+)-JQ1, a BET proteins inhibitor, in canine mammary cancer cells, suggesting BET proteins as a promising target to canine mammary cancer. (+)-JQ1 was the first inhibitor design to inhibit this family of epigenetic readers, but due to its short half-life in plasma, it has failed to clinical trials (FILIPPAKOPOULOS et al., 2010; MOYER, 2011). However, efforts have been made to improve (+)-JQ1 stability overcoming this limitation. Nanoparticles containing (+)-JQ1 were used against a triple-negative breast cancer xenograft model and reduced growth and vascularity of tumor. In addition, encapsulation of (+)-JQ1 in these nanoparticles improves stability and increased half-time of this compound in plasma (MAGGISANO et al., 2020). Furthermore, there are several BET

inhibitors that have entered in phase I or II tests for patients with solid tumors and hematologic malignancies such as GSK525762, CPI-0610, GS-5829, and GSK2820151 (STATHIS; BERTONI, 2018).

Finally, in the last chapter, we initiate the first experiments to test a fresh published dual BET/HDAC inhibitor, TW09, in human breast cancer. The first findings were encouraging and we intend to still continue with substantial steps to determine TW09 as a potential and innovative strategy to treat mammary cancer in both human and canine species. The next purpose is based on to study deeply the mechanisms responsible for the observed effects so far and to understand how dual BET/HDAC inhibition by TW09 can target these cancer cells, reducing cell viability and promoting cell death.

All compounds and therapeutic strategies we mentioned above are most available and can be tested in veterinary oncology as well. We believe that understand the epigenetic modifications in canine cancer, along with the use of that species to comparative studies can further promote the advances in this field. Furthermore, this can elucidate even more opportunities for treating the disease in dogs, further improving the life quality of our best friends.

References

FILIPPAKOPOULOS, P. et al. Selective inhibition of BET bromodomains. **Nature**, v. 468, n. 7327, p. 1067–1073, 2010. Disponível em: <http://dx.doi.org/10.1038/nature09504>.

MAGGISANO, V. et al. Nanoparticles loaded with the BET inhibitor JQ1 block the growth of triple negative breast cancer cells in vitro and in vivo. **Cancers**, v. 12, n. 1, p. 1–14, 2020.

MOYER, M. W. First drugs found to inhibit elusive cancer target. **Nature Medicine**, v. 17, n. 11, p. 1325, 2011.

STATHIS, A.; BERTONI, F. BET proteins as targets for anticancer treatment. **Cancer Discovery**, v. 8, n. 1, p. 24–36, 2018.

TOLCHER, A. W. et al. A phase 1 study of anti-TGFβ receptor type-II monoclonal antibody LY3022859 in patients with advanced solid tumors. **Cancer Chemotherapy and Pharmacology**, v. 79, n. 4, p. 673–680, 9 abr. 2017. Disponível em: <http://link.springer.com/10.1007/s00280-017-3245-5>.

ZHU, H. et al. A novel TGFβ trap blocks chemotherapeutics-induced TGFβ1 signaling and enhances their anticancer activity in gynecologic cancers. **Clinical Cancer Research**, v. 24, n. 12, p. 2780–2793, 2018.

APPENDIX A – SUPPLEMENTARY MATERIAL OF CHAPTER 1

Supplementary Table S1. DNA Methylation modifications associated with different types of canine cancer.

DNA Methylation and Canine Cancer			
Associated Epigenetic Modification	**Findings**	**Type of Tumor**	**Reference**
DNA Hypomethylation	Genomic hypomethylation has been observed in canine lymphoma and leukemia	Lymphoma; Leukemia	[14]
DNA Hypomethylation	Genome hypomethylation was found in grade III canine mast cell tumor, correlating with the aggressiveness of this type of cancer	Mast cell tumor	[18]
DNA Hypomethylation	Higher DNA global hypomethylation of circulating leukocytes in dogs with NHL in comparison with healthy dogs	Non-Hodgkin Lymphoma	[19]
DNA Hypomethylation	DNA hypomethylation observed in canine lung cancer and in metastatic osteosarcoma from the primary lung cancer	Lung cancer	[20]
DNA Hypermethylation	DNA hypermethylation in DLC1, a tumor suppressor gene	Non-Hodgkin Lymphoma and canine chronic lymphocytic leukemia	[23]
DNA Hypermethylation	DLC1 hypermethylation was associated with the malignant phenotype of NHL. However, hypermethylation of the DLC1 promoter was not associated with silencing of DLC1 expression and did not correlate with survival	Non-Hodgkin Lymphoma	[23,24]
DNA Hypermethylation	Hypermethylation of *TNF-α* in canine melanoma cells. The methylation status and the level of *TNF-α* expression were inversely correlated in canine melanoma cell lines and melanoma tissues	Melanoma	[25]

DNA Hypermethylation	DNA hypermethylation of microRNA-203 CpG islands, a tumor suppressor miRNA	Melanoma	[26]
DNA Hypermethylation	DNA Hypermethylation of *TFPI-2*	B-cell Lymphoma	[28]
DNA Hypermethylation	DNA hypermethylation of DAPK	B-cell Lymphoma	[29]
DNA Hypermethylation	DNA hypermethylation of p16	Lymphoid Tumor	[30]
DNA Hypermethylation	DNA hypermethylation of HOXD10, FGFR2, ITIH5, RASAL3	B-cell Lymphoma	[31,32]
DNA Hypermethylation	*DAPK* hypermethylation is a negative prognostic factor in canine high-grade B-cell Lymphoma.	B-cell Lymphoma	[33]
DNA Hyper/Hypomethylation	A heterogeneous pattern of DNA methylation observed with subsets of cases hypermethylated or hypomethylated when compared with healthy tissues.	Acute myeloid leukemia	[34]
DNA Hyper/Hypomethylation	CpGs hypermethylation located in CGIs that were unmethylated in normal peripheral blood mononuclear cells (PBMCs). CpG sites outside CGIs lose methylation in lymphoma cells compared to the healthy PBMCs.	Lymphoma	[35]
DNA Hypermethylation	*BRCA1* hypermethylation in mammary cancer. The rate of *BRCA1*-hypermethylated samples was very low (1/15 – 6.7%) making it difficult to conclude that *BRCA1* downregulation is a consequence of *BRCA1* promoter hypermethylation	Mammary cancer	[38]
DNA Hypermethylation	CGIs of the *ABCB1* gene were hypomethylated in dogs with lymphoma	Lymphoma	[41]
DNA Methylation	No variation in methylation patterns were found between *ERα*-positive canine mammary carcinomas and *ERα*-negative canine mammary carcinomas pointing to a difference of *ERα* regulation mechanisms between human and dogs	Mammary cancer	[44]

Supplementary Table S2. Histone modifications associated with different types of canine cancer.

Histone Modifications and Canine Cancer			
Associated Epigenetic Modification	**Findings**	**Type of Tumor**	**Reference**
Histone Deacetylation	Lower histone acetylation levels were associated with poor prognosis of the animals with urothelial carcinomas	Urothelial Carcinomas	[61]
Histone Methyltransferase Mutation	*SETD2* gene, a histone methyltransferase and an important tumor-suppressor, was found to be mutated in 21% of canine osteosarcoma samples.	Osteosarcoma	[62]
Histone Methyltransferase Mutation	*SETD2* somatic point mutations, deletions and chromosomal translocations in 42% of canine osteosarcoma samples.	Osteosarcoma	[63]
Histone Methyltransferase Overexpression	Overexpression of *EZH2* in different types of canine cancer	Lymphoma; melanoma; basal cell tumors; squamous cells carcinoma; prostate; and mammary	[64,65]
Histone Modification	Complex canine mammary carcinomas displayed a number of epigenetic dysregulations, such as downregulation of chromatin-modification genes or abnormally enriched activating histone modification H4-acetylation, and reduction in the repressive histone modification H3K9me3.	Mammary Cancer	[67]

Supplementary Table S3. miRNA modifications associated with different types of canine cancer.

miRNA Modifications and Canine Cancer			
Associated Epigenetic Modification	**Findings**	**Type of Tumor**	**Reference**
Abnormal miRNA expression	*miR-29* and *miR-29b* were upregulated in canine mammary cancer. *miR-15a* and *miR-16* are downregulated in canine ductal carcinomas while *miR-181b, -21, -29b*, and *let-7f* showed a significant upregulation in canine tubular papillary carcinomas	Mammary Cancer	[80]
Abnormal miRNA expression	*miR-9* is increased in high grade canine mast cell tumor compared to low grade and normal samples	Mast cell tumor	[81]
Abnormal miRNA expression	*miR-9* overexpression in osteosarcoma tumors and cell lines compared to normal osteoblasts and associated with metastatic phenotype	Osteosarcoma	[82]
Abnormal miRNA expression	22 miRNAs are differentially expressed in splenic hemangiosarcoma and normal spleens samples	Hemangiosarcoma	[83]
Abnormal miRNA expression	5 miRNAs upregulated and 14 downregulated in prostate cancer in comparison with normal prostate suggesting these miRs have diagnostic and miR-based therapeutic potential in prostate cancer	Prostate Cancer	[84]
Abnormal miRNA expression	Upregulation of the miR-17-92, miR-29 family, and miR-34a were associated with B cell lymphoma and upregulation of the miR-181 with T cell lymphoma. Upregulation of the miR-181 and downregulation of miR-29b and miR-150 were associated with inferior response to CHOP therapy and survival. Higher expression of miR-155 and miR-222 were negatively	Lymphoma	[85]

	associated with outcome in both B and T cell lymphoma		
Abnormal miRNA expression	miR-383 and miR-204 were potential oncomiRs that may be involved in regulating melanoma development by evading DNA repair and apoptosis	Melanoma	[87]
Abnormal miRNA expression	Upregulation of m*iR-181* and *miR-17-5p* in B- and T-cell lymphomas	Lymphoma	[88]
Abnormal miRNA expression	*cfa-miR-362, cfa-miR-155, cfa-miR-182*, and *cfa-miR-124* as strongly associated with the metastasizing class in uveal melanomas	Melanoma	[89]
Abnormal miRNA expression	Ten miRNA were validated and showed significant different expression in metastatic and non-metastatic mammary tumors	Mammary cancer	[90]
Abnormal miRNA expression	*miR-34a* associated with invasion ability in canine osteosarcoma cell lines	Osteosarcoma	[91]
Abnormal miRNA expression	Circulating *miRNA-214* is a good diagnostic marker in sarcomas, whereas circulating *miRNA-126* was high in different types of canine tumors	Different epitelial and non-epithelial canine cancers.	[94]
Abnormal miRNA expression	miR-214 and miR-216 have a strong potential to predict the outcome of canine appendicular osteosarcoma patients receiving amputation and chemotherapy	Osteosarcoma	[95]
Abnormal miRNA expression	Downregulation of circulating *Let-7g* in histiocytic sarcoma	Histiocytic Sarcoma	[96]

Abnormal miRNA expression	Studying the profile of circulating serum miRNAs in dogs with lymphoma, *let-7b, miR-223, miR-25, miR-92a* were reduced, whereas *miR-423a* levels were significantly increased in dogs with lymphoma compared to the controls	Lymphoma	[97]
Abnormal miRNA expression	*miR-103b* and *miR-16* as potential diagnostic urine biomarkers to bladder cancer	Bladder cancer	[98]
Abnormal miRNA expression	Mammary cancer cells shed exosomes that contained differentially expressed miRNAs in comparison with normal cells	Mammary Cancer	[99]
Abnormal miRNA expression	*miR-151, miR-8908a-3p, and miR-486* derived from exosomes demonstrated to be differently expressed between vincristine-sensitive and resistant lymphoma cell lines.	Lymphoma	[100]

Supplementary Table S4. Studies observing the effects of DNA methyltransferases inhibitors in different types of canine cancer.

DNA Methyltransferase inhibitors and Canine Cancer			
Inhibitor	**Findings**	**Type of Tumor**	**Reference**
5-Aza-C	Dogs with urothelial carcinoma were treated with subcutaneous 5-aza-C. Partial remission was achieved in 22%; 50% showed stable disease, whereas in 22% the cancer progressed.	Urothelial Carcinoma	[119]
5-Aza-dC	5-aza-dC induced a new apoptosis-inducing mechanism in melanoma cells, through demethylation and induction of cytotoxic cytokines such as TNF-α in *in vitro* and *in vivo* experiments.	Melanoma	[25]
5-Aza-C	5-aza-C reduced *in vitro* growth, invasion, tumorigenicity, mitochondrial activity and increased the susceptibility to apoptosis of mammary cancer cells from human, canine and feline species.	Mammary cancer	[120]
Zebularine	Zebularine promotes global demethylation of canine malignant lymphoid cells resulting in dose-dependent apoptosis.	Lymphoma	[121]

Supplementary Table S5. Studies observing the effects of histone deacetylase inhibitors in different types of canine cancer.

Histone Deacetylase Inhibitors and Canine Cancer			
Inhibitor	**Findings**	**Type of Tumor**	**Reference**
Vorinostat	Vorinostat reduced the viability and increased apoptosis in a dose-dependent manner besides decreasing phosphorylation in oncogenic pathways including Akt-Ser^{473} and mTOR in canine osteosarcoma cell lines	Osteosarcoma	[140]
Vorinostat	Inhibited the growth and induced G0/G1 cell cycle arrest through the upregulation of p21 and dephosphorylation of Rb in urothelial carcinoma cells	Urothelial Carcinoma	[61]
Sulforaphane	Decreased cell invasion and downregulating focal adhesion kinase (FAK) signaling in canine osteosarcoma cells	Osteosarcoma	[141]
Panobinostat	Inhibited B-cell Lymphoma xenograft tumor growth, triggering acetylation of H3 and apoptosis in *vivo*	Lymphoma	[142]
Panobinostat	Panobinostat efficiently inhibited the growth of tumors in xenograft models inoculated with a canine B-cell lymphoma cell line	Lymphoma	[143]
Trichostatin A	Trichostatin A decreased cell viability and increased apoptosis in canine grade 3 mast cell tumor	Mast cell tumor	[145]
Trichostatin A	Trichostatin inhibited the proliferation of one canine mammary cancer cell line	Mammary Cancer	[146]
AR-42	Induced cell viability inhibition and induction of apoptosis via activation of the intrinsic mitochondrial pathway were observed in canine osteosarcoma cells	Osteosarcoma	[140]

AR-42	Inhibited *in vitro* proliferation in a time- and dose-dependent manner and decreased migration and the incidence of bone metastasis in xenograft models	Prostate Cancer	[148]
AR-42	AR-42 in canine malignant mast cells induced proliferation inhibition, cell cycle arrest, apoptosis, and activation of caspases-3/7. Inhibition of KIT, p-AKT and STAT3/5 was also observed	Mast cells tumor	[149]
Valproic Acid (VPA)	A study of combined valproic acid (VPA) and doxorubicin was performed in spontaneous canine cancers. 10% presented complete responses, 14% presented partial responses, 24% showed stable disease after treatment, and 58% exhibited progressive disease	Lymphomas, melanoma, lung cancer, osteosarcoma, renal cell carcinoma, soft-tissue carcinoma.	[150]
Valproic Acid (VPA)	Pre-incubation with VPA followed by doxorubicin increased the growth inhibition and apoptosis rates in canine osteosarcoma cells	Osteosarcoma	[151]

Supplementary Table S6. Studies observing the effects of alternative epigenetic inhibitors in different types of canine cancer.

Alternative epigenetic inhibitors and Canine Cancer			
Inhibitor	**Findings**	**Type of Tumor**	**Reference**
(+)-JQ1	(+)-JQ1, a BET proteins inhibitor, decreased cell viability of canine mammary cancer cells. Furthermore, (+)-JQ1 was very efficient to inhibit colonies and tumorspheres formation, demonstrating an effect on tumorigenicity and self-renewal	Mammary cancer	[159]
FTY720	FTY720, a SET methyltransferase inhibitor, suppressed cell proliferation, colony formation, and *in vivo* tumor growth of canine mammary and osteosarcoma cell lines. Furthermore, SET knockdown repressed mTOR and NF-kB signaling in both types of canine cancer	Osteosarcoma; Mammary cancer	[160,161]
BB-Cl-Amidine	BB-CLA, an inhibitor of protein-arginine deiminases (PADs), resulted in the decrease of viability and tumorigenicity of canine mammary cancer cells, activating endoplasmic reticulum stress pathway in these cells	Mammary cancer	[162]
GSK2879552	GSK2879552, a LSD1 inhibitor, caused severe but reversible toxicities in dogs including thrombocytopenia, neutropenia, myelofibrosis, and congestion with and without lymphoid necrosis in lymphoid organs	Healthy dogs used as preclinical models	[155]

APPENDIX B – SUPPLEMENTARY MATERIAL OF CHAPTER 2

Supplementary Table S1: Ratio 260/280 nm and 260/230 nm of the RNA samples.

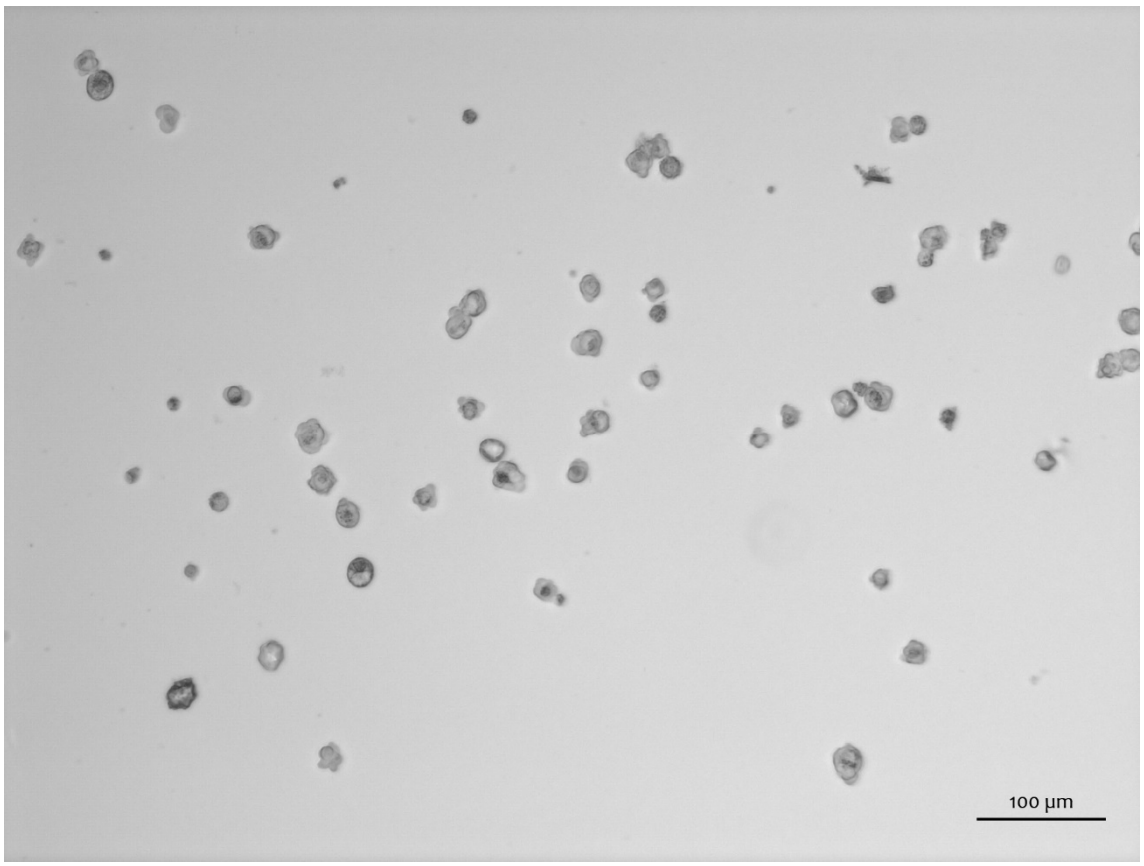
Samples	260/280	260/230
M5-1	1.94	1.98
M5-2	1.83	2.1
M5-3	1.91	1.97
E20-1	1.93	2.1
E20-2	1.99	1.63
E20-3	1.93	2.2
M25-1	1.92	1.99
M25-2	1.91	2.0
M25-3	1.93	2.0
E37-1	1.93	2.1
E37-2	2.0	1.66
E37-3	1.94	2.2
CF41.Mg-1	1.94	2.2
CF41.Mg-2	1.94	2.13
CF41.Mg-3	2.0	1.81

Supplementary Table S2. Gene-specific primer sequences used for real time quantitative PCR.

Primers	Sequence
18S (NR_003278.3)	F: 5'-CCTGCGGCTTAATTTGACTC-3' R: 5'-CTGTCAATCCTGTCCGTGTC-3'
CDH1 (NM_001287125.1)	F:5'- AGGCCCATTTCCTAAAAACC -3' R:5'- TTGGCCAGTGATGCTGTAGA -3'
SLUG (NM_001097981.1)	F: 5'-CGTTTTCCAGACCCTGGTTA-3' R: 5'-GCAGTGAGGGCAAGAAAAAG-3'
STAT3 (XM_005624457.2)	F: 5'-GCCAATGCTAGAGGAGAGGA-3' R: 5'-AACTGGACGCCAGTCTTGAT-3'
ZEB1 (XM_005617037.2)	F: 5'-TTGCTCCCTGTGCAGTTACA-3' R: 5'-AGCTTTCCCACATTCAGTGC-3'
ZEB2 (XM_005631964.2)	F: 5'-ACCCAGGACTGGATCAGATG-3' R: 5'-GCTCCATCAAGCAATTCTCC-3'

Supplementary Table S3. Alignment rates of reads from RNA expression of canine mammary cancer cells with the canine genome.

Samples	Number of input reads	Average input read length	Uniquely mapped reads number	% Uniquely mapped reads	Number of reads mapped to multiple loci	% of reads mapped to multiple loci	Number of reads mapped to too many loci	% of reads mapped to too many loci	% of reads unmapped: too short
E20-A	28988456	152	25462533	87.84	1229540	4.24	59167	0.20	7.66
E20-B	36699515	152	32434632	88.38	1573701	4.29	72836	0.20	7.08
E20-C	34156485	152	30196744	88.41	1422883	4.17	67420	0.20	7.17
M5-A	17605245	100	14794559	86.4	840021	5.7	374060	2.5	4.51
M5-B	15865311	100	13313857	86.3	831164	6.2	306422	2.3	3.67
M5-C	15586596	100	12459555	82.4	914089	7.3	273252	2.2	3.85
M25-A	32164176	152	28729376	89.32	987284	3.07	30419	0.09	7.48
M25-B	39007922	152	35276361	90.43	1201705	3.08	36365	0.09	6.36
M25-C	36884144	152	33185709	89.97	1148398	3.11	35448	0.10	6.79
CF41-A	38088440	152	34057219	89.42	1559246	4.09	68546	0.18	6.27
CF41-B	35675796	152	31787275	89.10	1456856	4.08	63320	0.18	6.59
CF41-C	33570363	152	28006290	83.43	1477156	4.4	65236	0.19	11.93



Supplementary Figure S1. **Tumorsphere formation assay of E37 cell line.** E37 cells were not able to generate tumorspheres in low-adherent plates.

APPENDIX C – SUPPLEMENTARY MATERIAL OF CHAPTER 3

Supplementary Table S1: Concentration, 260/280 nm and 260/230 nm ratio of the RNA samples.

Samples	RNA Concentration (ng/µl)	260/280	260/230
C1	434.3	1.95	2.2
C2	537.9	1.94	2.3
C3	405	1.93	2.3
J1	275.5	1.98	1.9
J2	269.9	1.95	2.2
J3	233.4	1.95	2.1

Supplementary Table S2. Gene-specific primer sequences used for real time quantitative PCR.

Primers	Sequence
18S (NR_003278.3)	F: 5'-CCTGCGGCTTAATTTGACTC-3' R: 5'-CTGTCAATCCTGTCCGTGTC-3'
BRD2 (NM_001048087.1)	F:5'-GGAGTTTGCTGCTGATGTGC-3' R:5'-CCCTGGTTCCAGTGGTTCAT-3'
BRD3 (XM_005625157.3)	F:5'-GCAATCAAGCTGAACCTGCC-3' R:5'-GTCCTGCATACATTCGCTCG-3'
BRD4 (XM_014122040.2)	F:5'-GCCACCGTTCCAGAGCC-3' R:5'-TCCTCCTTCACCACCACCA-3'
CDH1 (NM_001287125.1)	F: 5'-AGGCCCATTTCCTAAAAACC-3' R: 5'-TTGGCCAGTGATGCTGTAGA-3'
c-Myc (NM_001003246.2)	F:5'-TTTGGACGCTGGATCTCCTC-3' R:5'-AAGCTGACGTTGAGAGGCAT-3'
SLUG (NM_001097981.1)	F: 5'-CGTTTTCCAGACCCTGGTTA-3' R: 5'-GCAGTGAGGGCAAGAAAAAG-3'
SOX2 (XM_005639752.3)	F: 5'-GTCCCAGCACTACCAGAGCG-3' R:5'-CTTACTCTCCTCCCATTTCCCTCG-3'
STAT3 (XM_005624457.2)	F: 5'-GCCAATGCTAGAGGAGAGGA-3' R: 5'-AACTGGACGCCAGTCTTGAT-3'

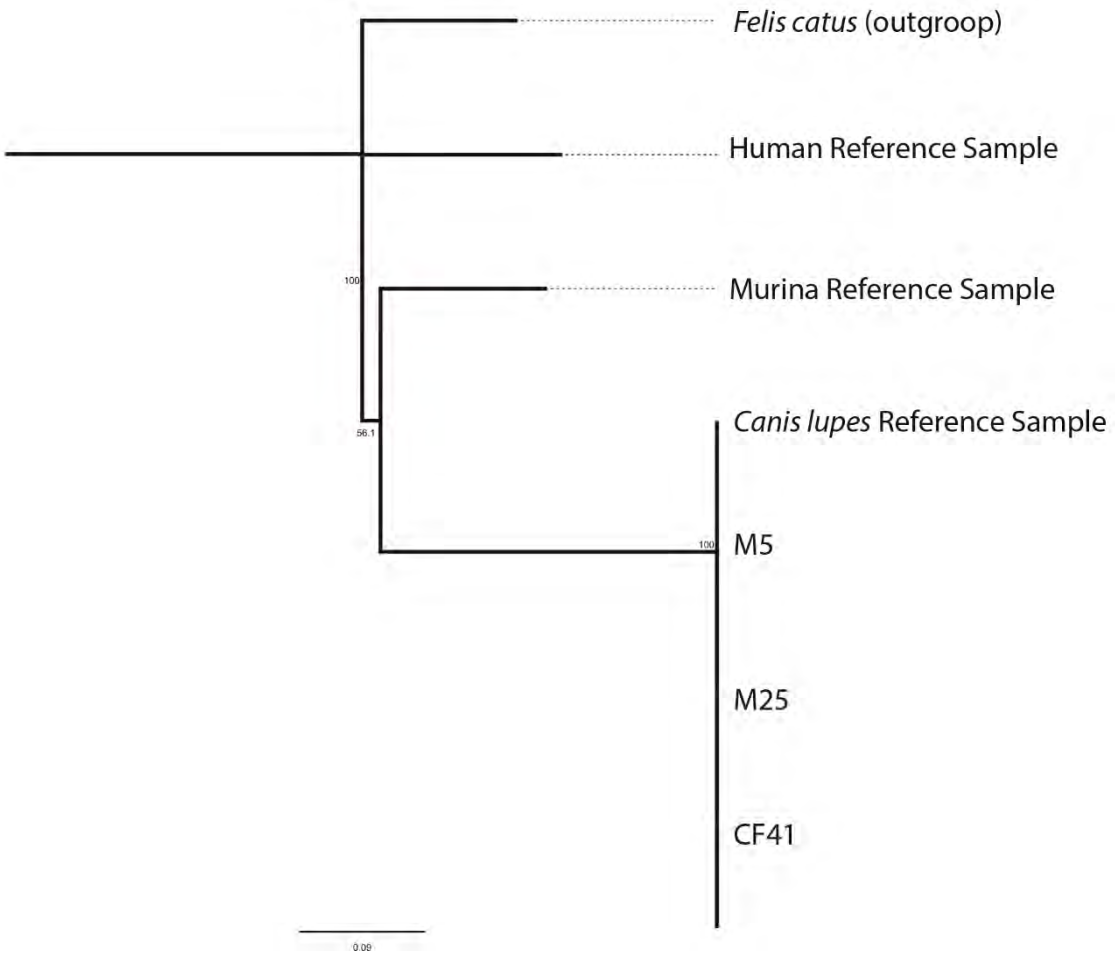
ZEB1 (XM_005617037.2)	F: 5'-TTGCTCCCTGTGCAGTTACA-3' R: 5'-AGCTTTCCCACATTCAGTGC-3'
ZEB2 (XM_005631964.2)	F: 5'-ACCCAGGACTGGATCAGATG-3' R: 5'-GCTCCATCAAGCAATTCTCC-3'

Supplementary Table S3. RNA concentration and RNA integrity number (RIN) of control tumorspheres (C) and treated with 100 nM (+)-JQ1 (J).

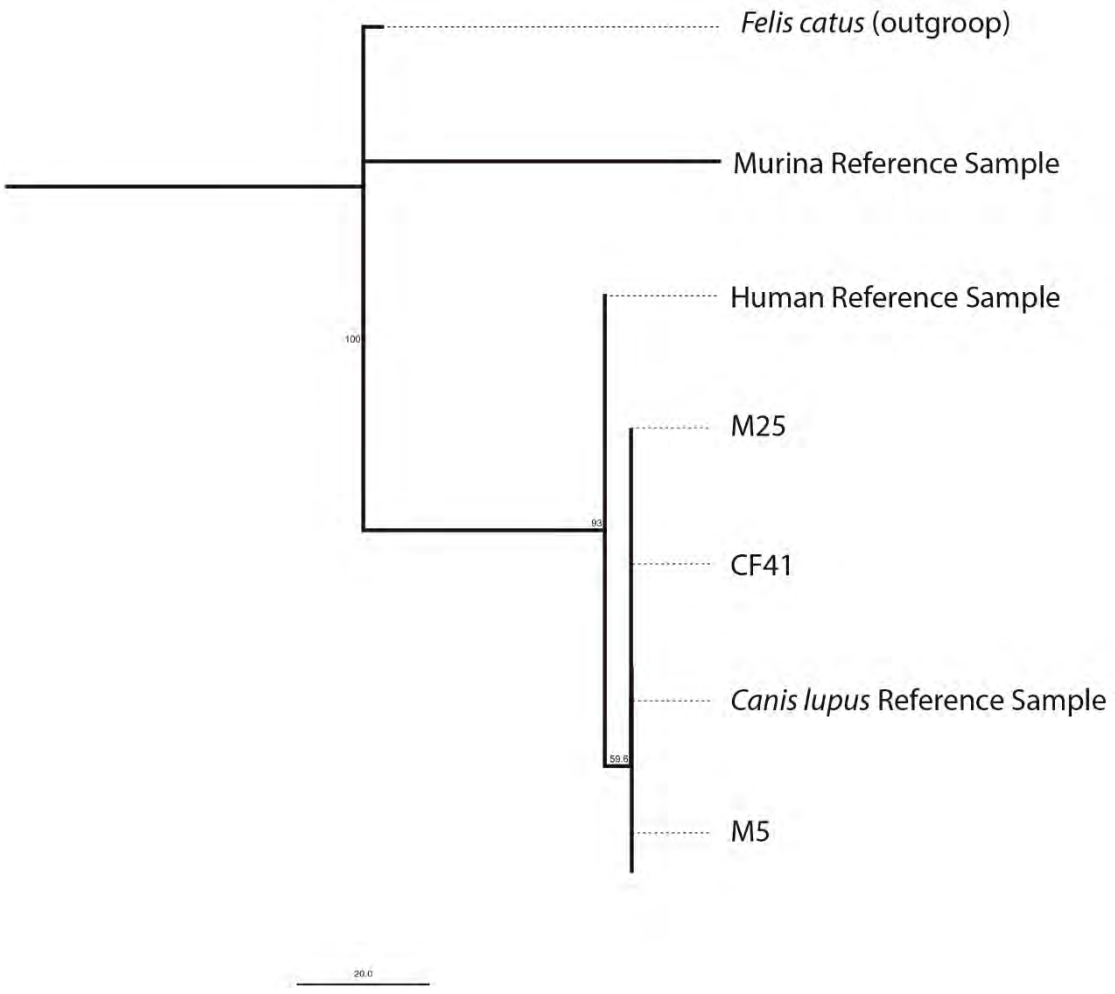
Samples	Concentration (ng/µl)	RIN
C1	326	10
C2	346	10
C3	426	10
J1	117	10
J2	152	10
J3	157	9.7

Supplementary Table S4. Alignment rates of control (C) and (+)-JQ1-treated tumorspheres (J).

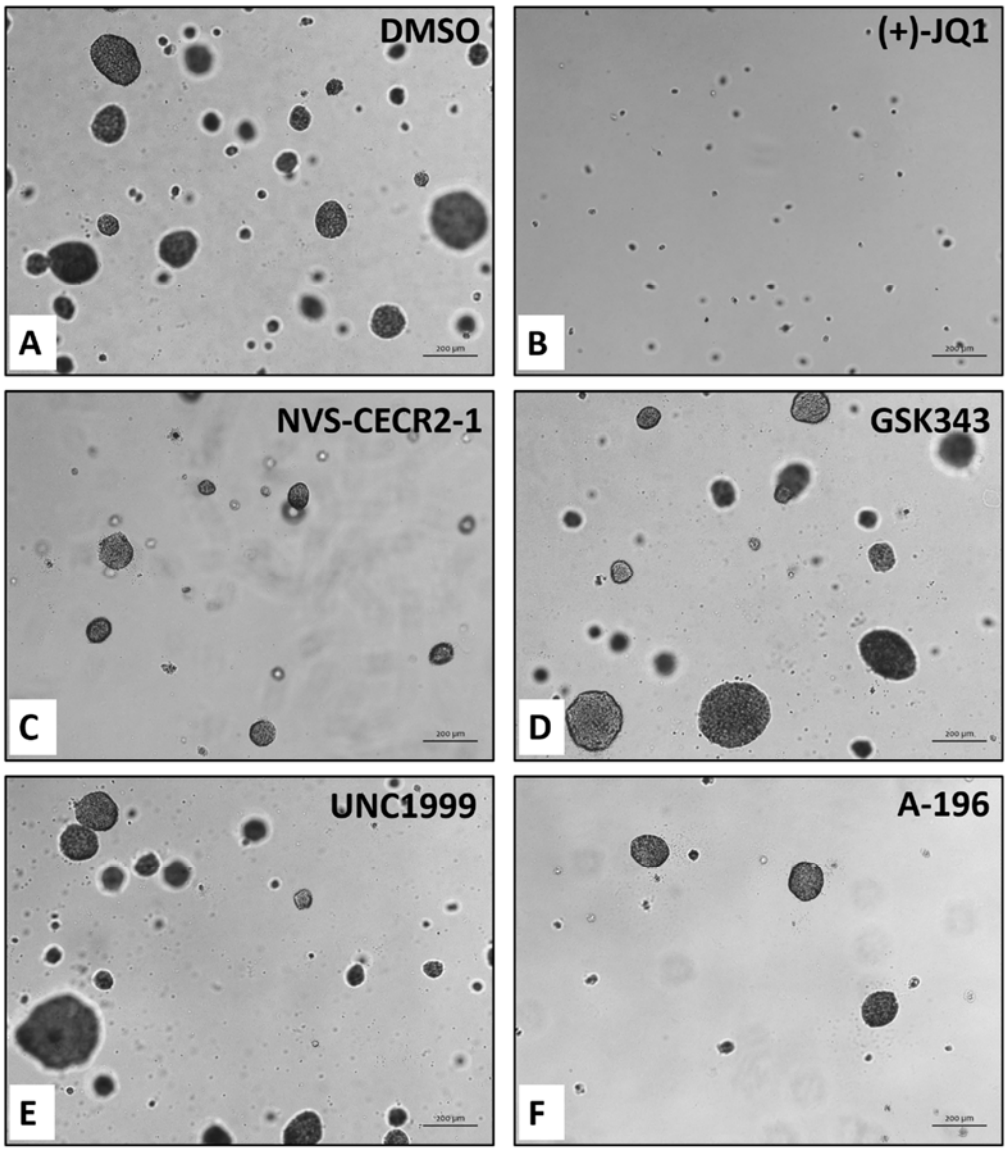
Samples	Number of input reads	Average input read length	Uniquely mapped reads number	% Uniquely mapped reads	Number of reads mapped to multiple loci	% of reads mapped to multiple loci	Number of reads mapped to too many loci	% of reads mapped to too many loci	% of reads unmapped: too short
C1	21134309	198	19250789	91.09	1127917	5.34	54134	0.26	3.25
C2	18900257	199	17242791	91.23	1001814	5.30	47513	0.25	3.16
C3	19065644	199	17392134	91.22	1039665	5.45	47738	0.25	3.01
J1	18531549	199	16564896	89.39	1077535	5.81	43377	0.23	4.51
J2	21907957	198	19824356	90.49	1208771	5.52	57637	0.26	3.67
J3	19379659	198	17441950	90	1131391	5.84	48753	0.25	3.85



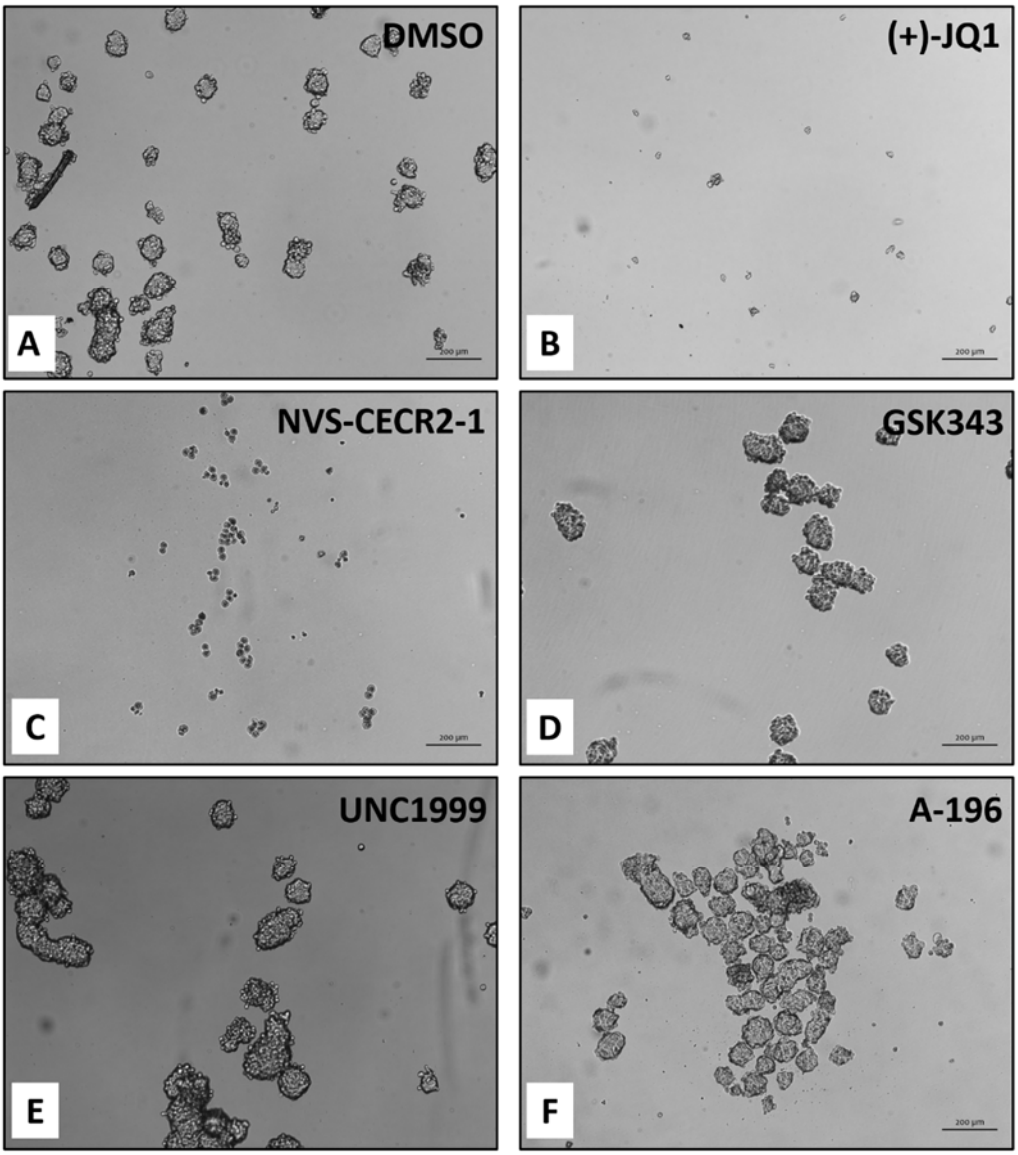
Supplementary Figure S1. COI Neighbor Joining tree of cell lineage confirmation. M5, M25 and CF41.Mg represents cell lineages evaluated. The *Canis Lupus* reference sample DNA was obtained from leukocytes of a known dog (*Canis lupus familiaris*). The human and murine reference DNA samples were obtained from H460 human lung cancer cell line and E9 murine cell, respectively. *Felis catus* (outgroup) sequence obtained from GenBank (accession number J958339.1).



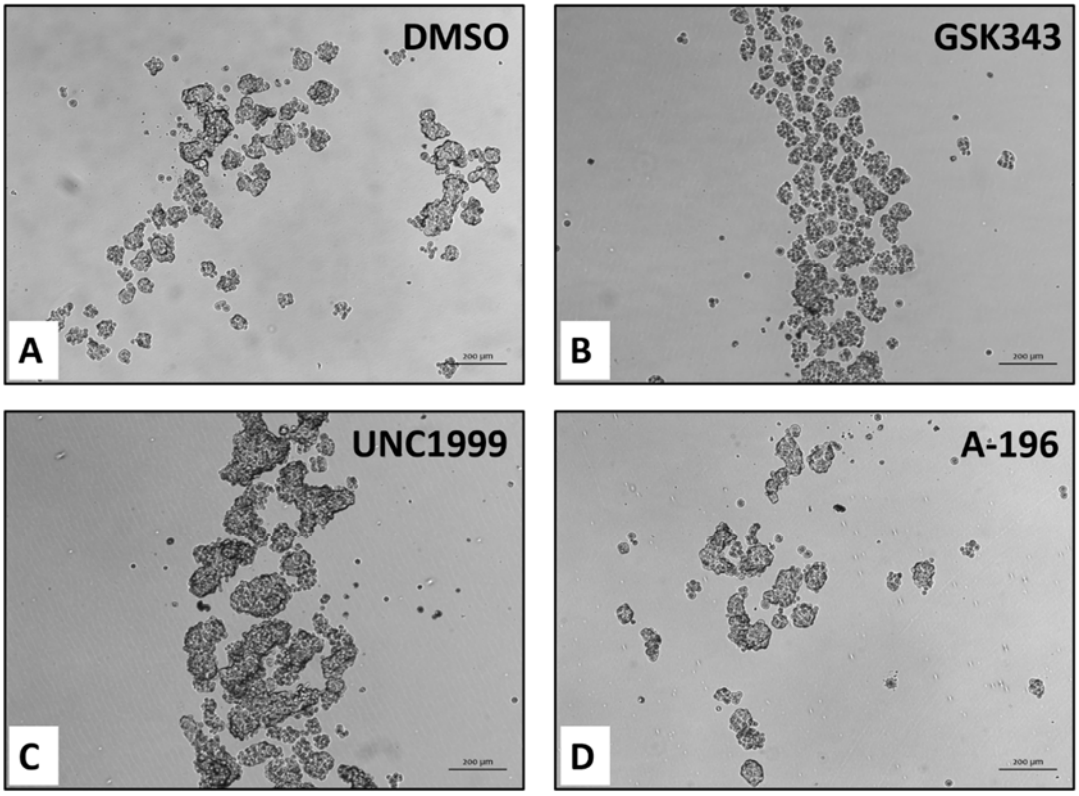
Supplementary Figure S2. 16S Neighbor Joining tree of cell lineage confirmation. M5, M25 and CF41.Mg represents cell lineages evaluated. The *Canis Lupus* reference sample DNA was obtained from leukocytes of a known dog (*Canis lupus familiaris*). The human and murine reference DNA samples were obtained from H460 human lung cancer cell line and E9 murine cell, respectively. *Felis catus* (outgroup) sequence obtained from GenBank (accession number DQ983942).



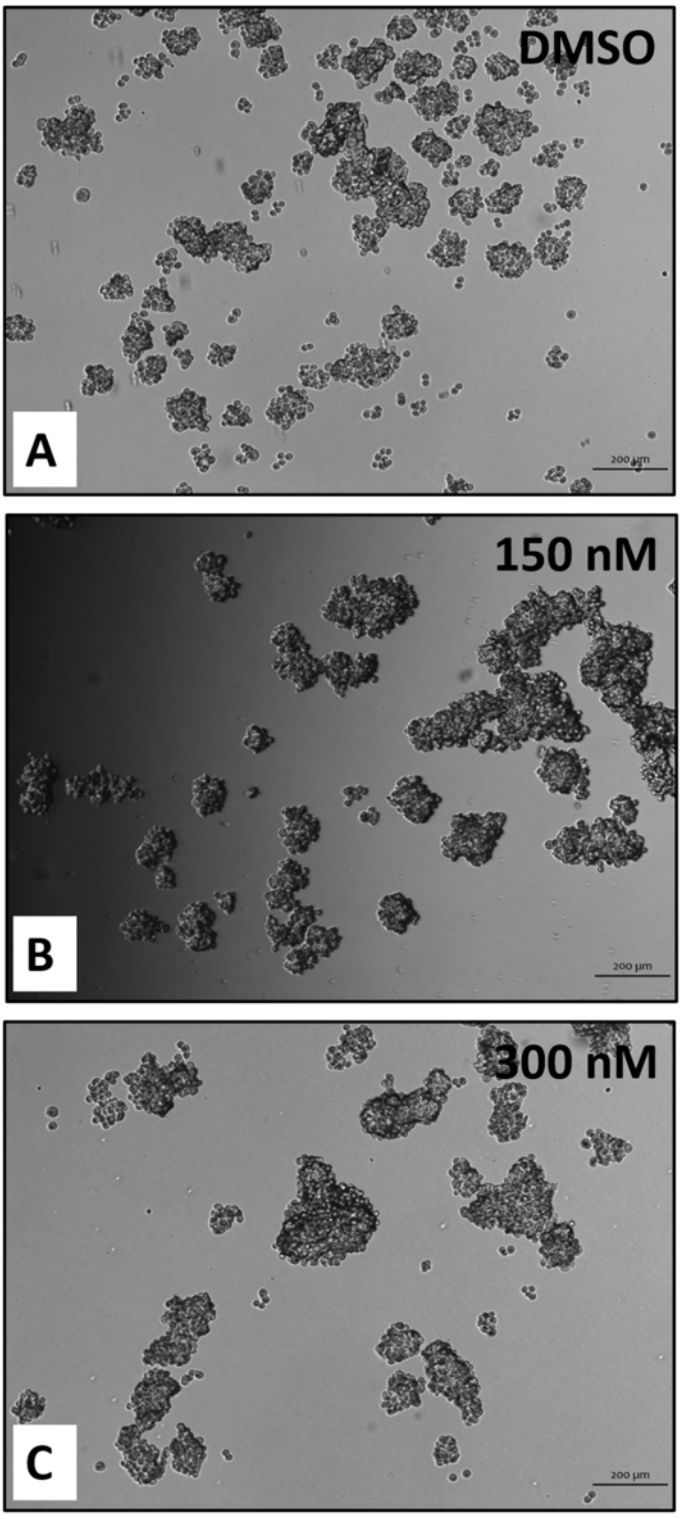
Supplementary figure S3. Effects of 27 epigenetic inhibitors regarding colonies formation using soft agar assay. Only (+)-JQ1 (**B**), NVS-CECR2-1 (**C**), GSK343 (**D**), UNC1999 (**E**) and A-196 (**F**) decreased the number of colonies in comparison to the control (DMSO) (**A**). Images were obtained in a 5x objective.



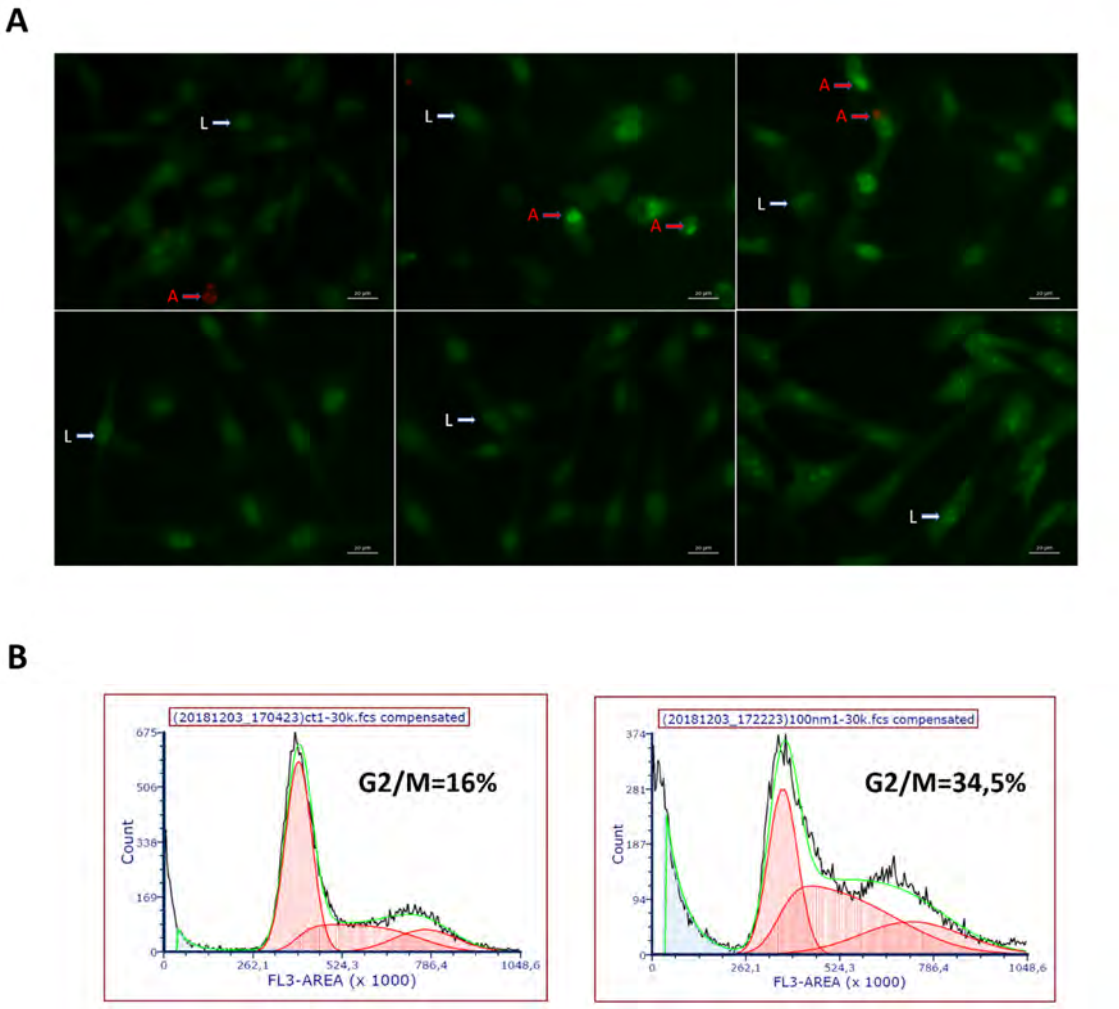
Supplementary Figure S4. Effects of (+)-JQ1, NVC-CECR2-1, GSK343, UNC1999, and A-196 regarding tumorspheres formation using low-adhrent plates. Only (+)-JQ1 (**B**) and NVS-CECR2-1 (**C**) were able to inhibit the tumorsphere formation in low-adherent plates. No difference was observed to tumorspheres treated with GSK343 (**D**), UNC1999 (**E**), and A-196 (**F**) in comparison to the control (DMSO) (**A**). Images were obtained in a 5x objective.



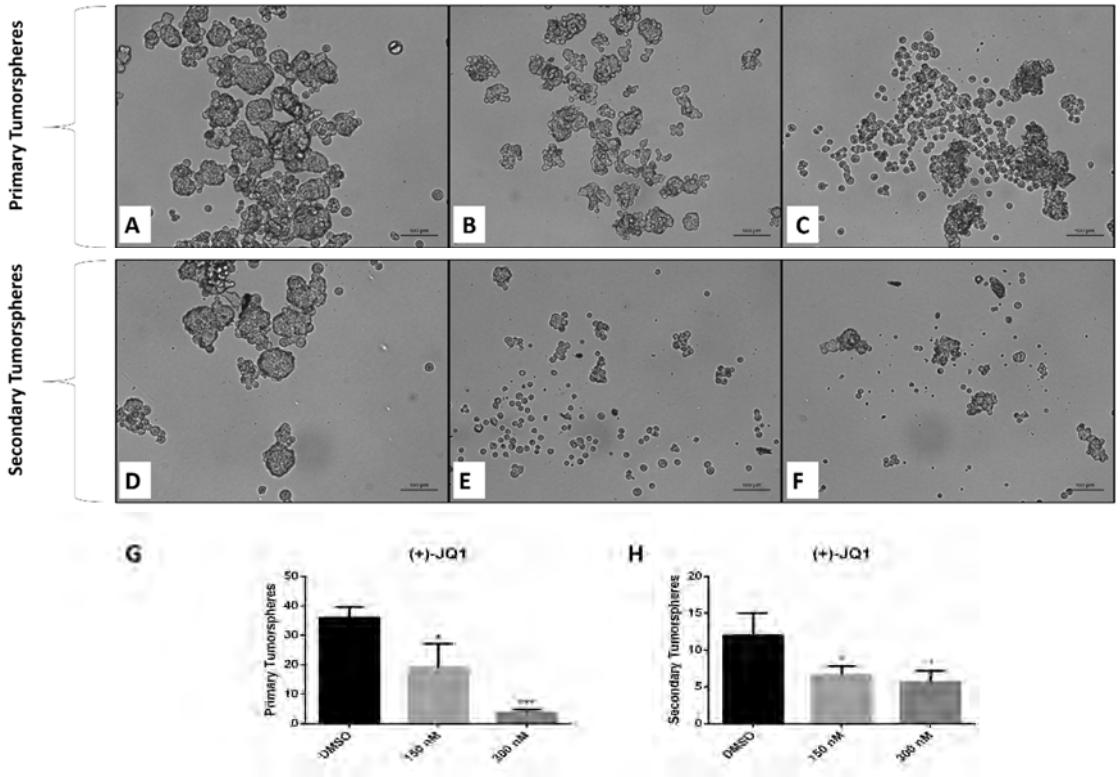
Supplementary Figure S5. Effects of GSK343, UNC1999, and A-196 on the formation of secondary tumorspheres using low-adherent plates. GSK343 (**B**), UNC1999 (**C**) and A-196 (**D**) were also unable to inhibit the formation of secondary tumorspheres in comparison to the control (**A**). Images were obtained in a 5x objective.



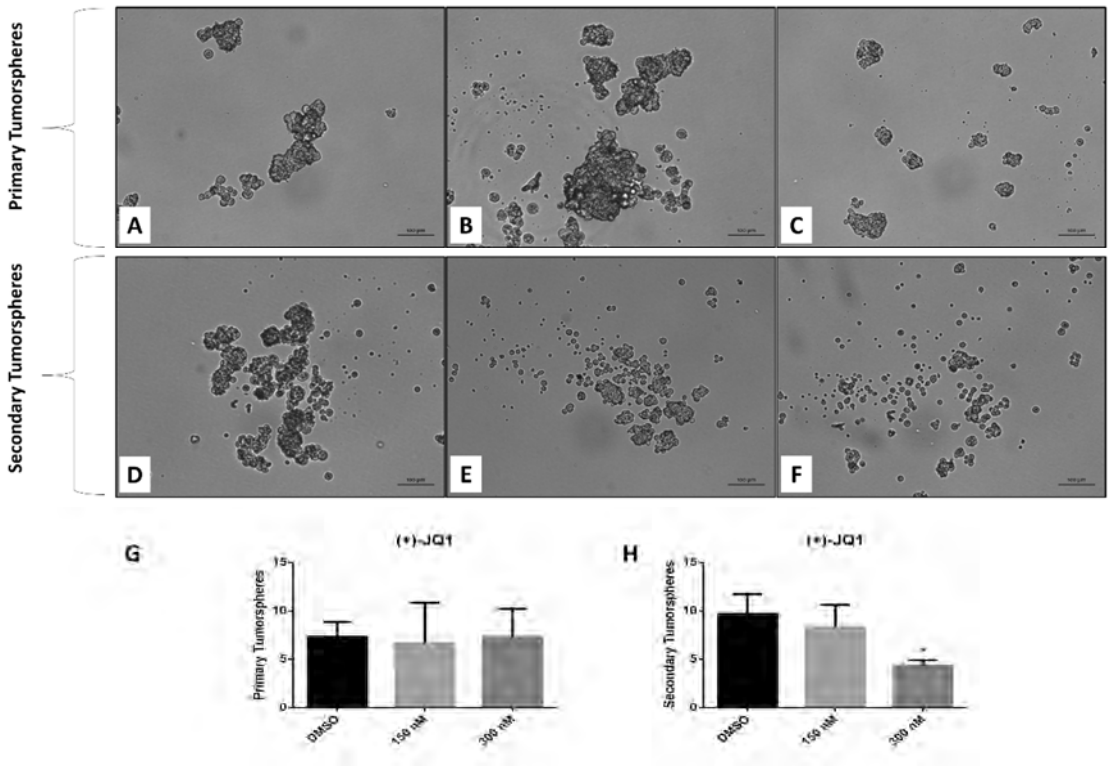
Supplementary figure S6. Effects of lower doses of NVS-CECR2-1 regarding tumorspheres formation using low-adherent plates. Lower doses of 150 nM (**B**) and 300 nM (**C**) NVS-CECR2-1 did not show the same effect on CF41.Mg tumorspheres in comparison to the control (**A**). Images were obtained in a 5x objective.



Supplementary Figure S7. Effects of (+)-JQ1 regarding cell death and cycle of CF41.Mg cell line. (A) Four µM or 1 µM, respectively of (+)-JQ1 induced apoptosis in CF41.Mg cells. On the other hand, 300 nM, 150 nM and 100 nM of (+)-JQ1 showed no difference in comparison to the control (L= Live cells; A= Apoptotic cells). **(B)** Flow cytometry histograms for CF41.Mg cells show increase G2/M cell cycle arrest in (+)-JQ1 treated cells compared to the control.



Supplementary Figure S8. Effects of lower doses of (+)-JQ1 on tumorsphere formation of M5 cell line. (+)-JQ1 at concentrations of 150 nM (**B, E**) and 300 nM (**C, F**) reduced the number of primary and secondary tumorspheres of M5 cells in comparison to the control (DMSO) (**A, D**). Images were obtained in a 5x objective. (*p< 0.05; *** p <0.001 - One way ANOVA followed by Tukey's multiple comparison test).



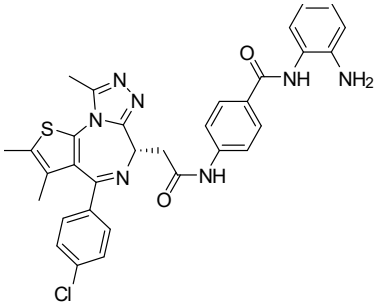
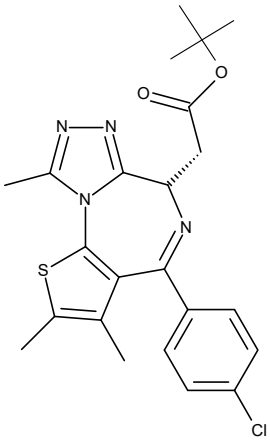
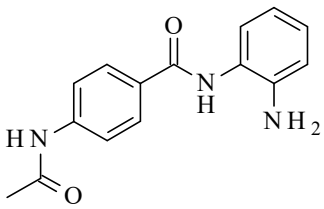
Supplementary figure S9. Effects of lower doses of (+)-JQ1 on tumorsphere formation of M25 cell line. On M25 cells, only the number of secondary tumorspheres was reduced, when treated with 300 nM (+)-JQ1 (**F**). Primary tumorspheres number was not affected by low concentrations of (+)-JQ1 (**B, C**) in comparison to the control (DMSO) (**A**). The concentration of 150 nM of (+)-JQ1 was not able to decrease the number of secondary tumorspheres (**E**) in comparison to the control (DMSO) (**D**). Images were obtained in a 5x objective. (*p< 0.05 - One way ANOVA followed by Tukey's multiple comparison test).

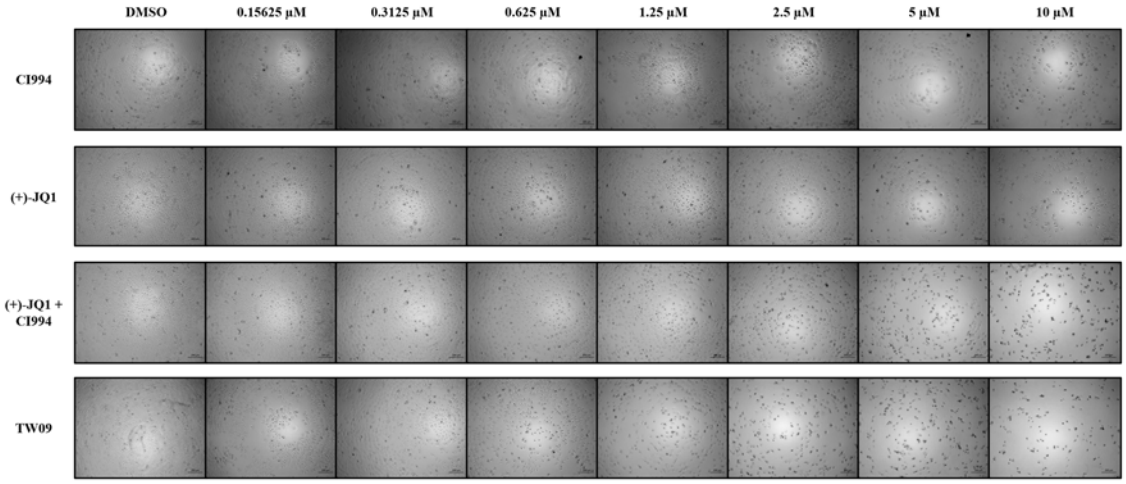
APPENDIX D – SUPPLEMENTARY MATERIAL OF CHAPTER 4

Supplementary Table 1. Gene-specific primer sequences used for real time quantitative PCR.

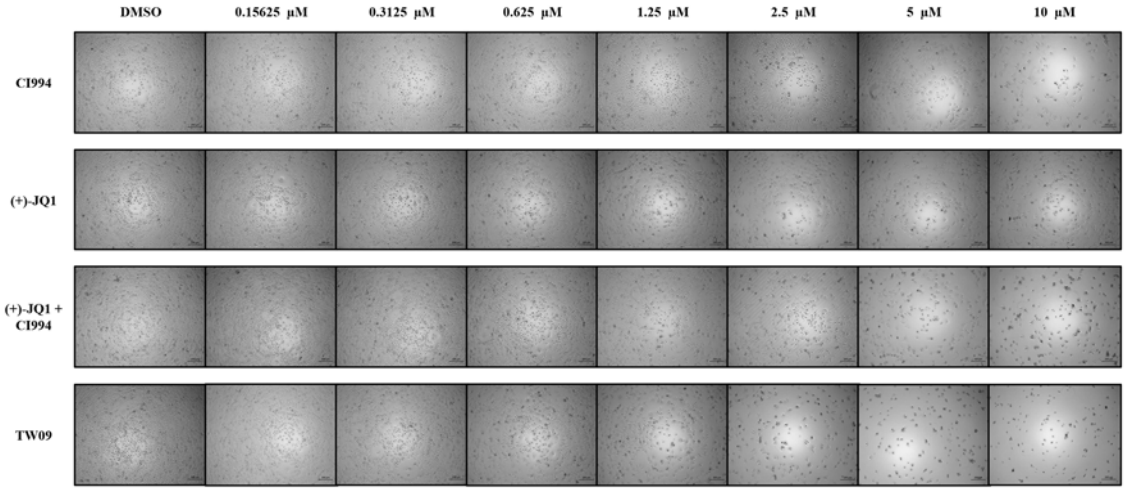
Primers	Sequence
RPLP0 (NM_001002.4)	F: 5'- AGCCCAGAACACTGGTC -3' R: 5'- ACTCAGGATTTCAATGGT -3'
BRD4 (NM_001330384.2; NM_014299.2; NM_058243.2)	F:5'- AGGCAAAAGGAAGAGGA -3' R:5'- CGATGCTTGAGTTGTGTT -3'
HDAC1 (NM_004964.3)	F:5'- TGACGAGTCCTATGAGGCCA -3' R:5'- CACTGTAAGACCACCGCACT -3'
c-Myc (NM_001354870.1; NM_002467.6)	F:5'- GCTGCTTAGACGCTGGATTT -3' R:5'- TAACGTTGAGGGGCATCG -3'

Supplementary Table 2. Description of the compounds used in this project.

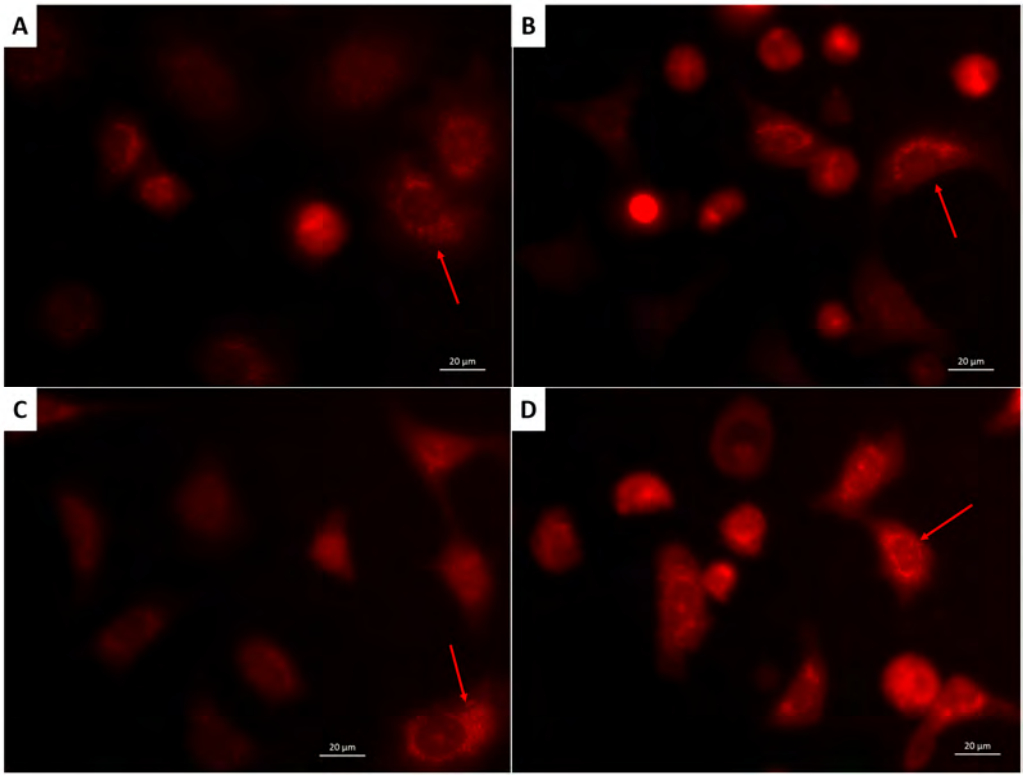
Compound	Structure	Molecular Weight
TW09	 TW09	609,17 g/mol
(+)-JQ1		456.1 g/mol
Tacedinaline (CI994)		269.3 g/mol



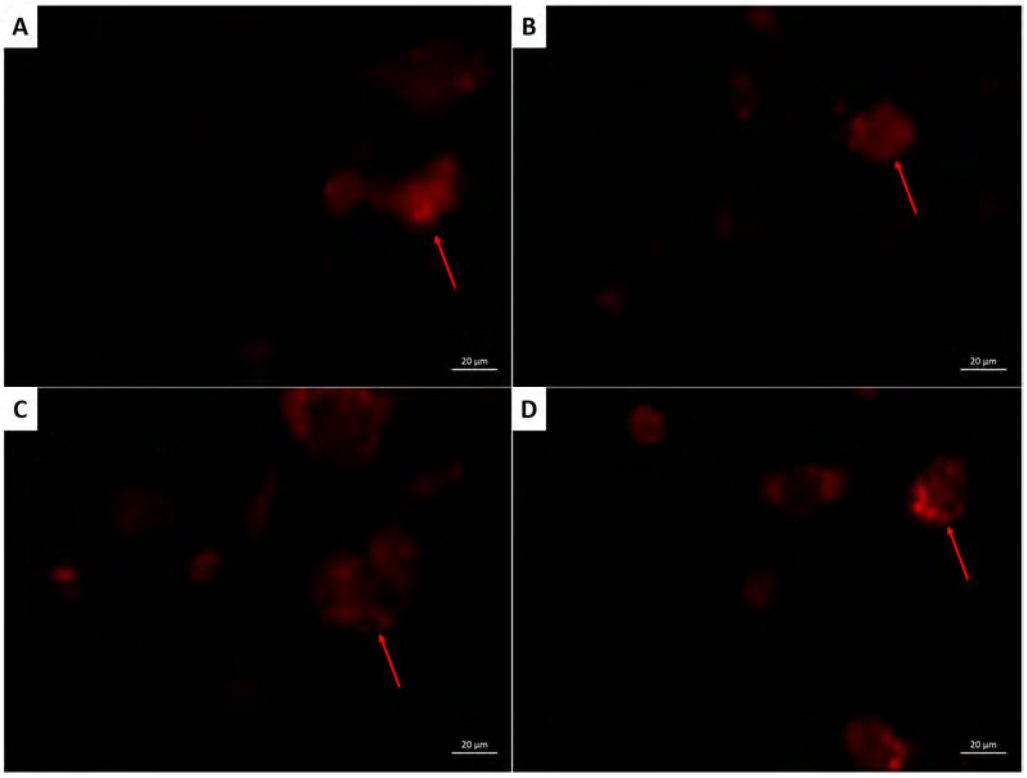
Supplementary Figure S1. Effects of different concentrations of CI994, (+)-JQ1, (+)-JQ1+CI994, and TW09 in MCF7 cell viability. (+)-JQ1 + CI994 and TW09 showed a stronger potential to reduce cell viability in MCF7 cells in comparison with (+)-JQ1 and CI994 individually.



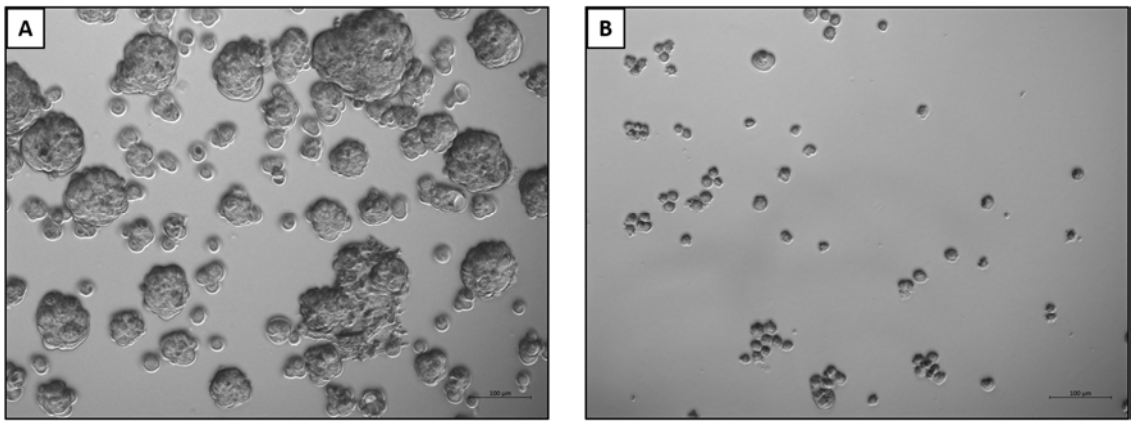
Supplementary Figure S2. Effects of different concentrations of CI994, (+)-JQ1, (+)-JQ1+CI994, and TW09 in MDA-MB-231 cell viability. (+)-JQ1 + CI994 and TW09 showed a stronger potential to reduce cell viability in MCF7 cells in comparison with (+)-JQ1 and CI994 individually.



Supplementary Figure S3. Staining of acidic vesicles with AO in MCF7 cells. Cells were treated with CI994 (**A**), (+)-JQ1 (**B**), (+)-JQ1 + CI994 (**C**), and TW09 (**D**).



Supplementary Figure S4. Staining of acidic vesicles with AO in MDA-MB-231 cells. Cells were treated with CI994 (**A**), (+)-JQ1 (**B**), (+)-JQ1 + CI994 (**C**), and TW09 (**D**).



Supplementary Figure S5. BC tumorspheres formation in low-adherent plates. Only MCF7 cells (**A**) were able to generate tumorspheres in low-adherent plates and in the described conditions. MDA-MB-231 cells (**B**) died or formed cell aggregates in low-adherent plates. The magnification of the images is 100X.