

**CARACTERIZAÇÃO DE RNAs LONGOS NÃO
CODIFICANTES EM ORGANOIDES DERIVADOS DE
CARCINOMA SEROSO DE OVÁRIO**

NAIADE CALANCA

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**CARACTERIZAÇÃO DE RNAs LONGOS NÃO
CODIFICANTES EM ORGANOIDES DERIVADOS DE
CARCINOMA SEROSO DE OVÁRIO**

Candidata: Naiade Calanca

Orientadora: Profa. Titular Dra. Silvia Regina Rogatto

Coorientadora: Profa. Dra. Cláudia Aparecida Rainho

Tese apresentada ao Instituto de Biociências,
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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE NAIADÉ CALANCA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA), DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 03 dias do mês de julho do ano de 2024, às 13:30 horas, no(a) Sala da Superintendência do HC-FMB-UNESP, realizou-se a defesa de TESE DE DOUTORADO de NAIADÉ CALANCA, intitulada **Caracterização de RNAs longos não codificantes em organoides derivados de carcinoma seroso de ovário**. A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra. SILVIA REGINA ROGATTO (Orientador(a) - Participação Presencial) do(a) Núcleo de Laboratórios de Análises Clínicas / Hospital das Clínicas da Faculdade de Medicina de Botucatu - UNESP, Profa. Dra. NADIA APARECIDA BERGAMO (Participação Virtual) do(a) Centro de Genética Humana / Instituto de Ciências Biológicas - Universidade Federal de Goiás, Prof. Dr. ROBSON FRANCISCO CARVALHO (Participação Virtual) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP, Profa. Dra. FLAVIA KARINA DELELLA (Participação Presencial) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP, Research Assistant Associate HELLEN KUASNE (Participação Virtual) do(a) Rosalind and Morris Goodman Cancer Institute / McGill University (Montreal, Canadá). Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: APROVADA. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. SILVIA REGINA ROGATTO



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Dedicatória

À minha tia e madrinha, Maria Salete Bueno Silveira (*in memoriam*),
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Epígrafe

*“The highest education is that which does not merely give us information but makes
our life in harmony with all existence.”*

*“A educação mais elevada é aquela que não apenas nos dá informação, mas que
coloca a nossa vida em harmonia com toda a existência.”*

— Rabindranath Tagore

(tradução livre)

Resumo

INTRODUÇÃO O carcinoma seroso de ovário (SOC) é o subtipo mais comum de câncer de ovário (OC) e uma das principais causas de morte entre os tumores ginecológicos. A sua natureza assintomática leva ao diagnóstico em estágios avançados (~75% dos casos), o que resulta em um prognóstico desfavorável. Inicialmente, as pacientes respondem à quimioterapia, contudo estima-se que 80% dos tumores avançados recidivam. Os RNAs longos não codificantes (lncRNAs) têm sido implicados na progressão e na resposta à terapia de vários tumores, incluindo o OC. **OBJETIVOS** Caracterizar a expressão de lncRNAs e investigar seu envolvimento na resposta à carboplatina em linhagens celulares de OC e organoides derivados de SOC; e buscar alvos acionáveis por drogas epigenéticas (*epi-drugs*) que possam interferir na resposta à quimioterapia. **MÉTODOS E PACIENTES** Os organoides derivados de tumor (TDOs) foram estabelecidos a partir de amostras de efusões malignas (efusão pleural ou ascite) coletadas de 15 pacientes com SOC submetidas a diferentes regimes terapêuticos. Dados de RNA-Seq dos TDOs, de sete amostras de tecido ovariano normal e de linhagens de OC sensíveis e resistentes à carboplatina foram obtidos e analisados. Estes dados foram comparados com dados externos de RNA-Seq de organoides derivados de SOC, de amostras de SOC (*The Cancer Genome Atlas*) e de tecido ovariano normal (*The Genotype-Tissue Expression*). Bancos de dados públicos foram consultados para filtrar genes diferencialmente expressos envolvidos com a regulação epigenética e prever interações entre seus produtos. LncRNAs potencialmente envolvidos com a maquinaria epigenética foram investigados e selecionados para ensaios funcionais utilizando as linhagens celulares de OC resistentes à carboplatina. **RESULTADOS** Identificamos lncRNAs, incluindo *SNHG12* e *MEG3*, e genes codificadores de proteínas potencialmente envolvidos na regulação epigenética diferencialmente expressos nas linhagens e nos TDOs. A expressão anormal desses lncRNAs foi confirmada nos conjuntos de dados externos. Nas linhagens resistentes, o aumento da resposta à carboplatina foi verificado após o silenciamento de *SNHG12*. Além disso, após o tratamento com a *epi-drug* decitabina, sozinha ou em combinação com tazemetostate, as linhagens celulares e o TDO testados mostraram maior sensibilidade à carboplatina e aumento dos níveis de expressão de *MEG3* nas linhagens celulares. **CONCLUSÃO** Identificamos lncRNAs desregulados no SOC que são capazes de interagir com efetores epigenéticos e são alvos promissores para reverter a quimiorresistência.

Abstract

BACKGROUND Serous ovarian carcinoma (SOC) is the most common subtype of ovarian cancer (OC) and one of the leading causes of death among gynecological tumors. Its asymptomatic nature leads to diagnosis at advanced stages (~75% of cases), which results in a poor prognosis. Although patients are initially responsive to chemotherapy, it is estimated that 80% of advanced-stage tumors will recur. Long noncoding RNAs (lncRNAs) have been implicated in progression and response to therapy in several tumor types, including OC.

OBJECTIVES To characterize the expression of lncRNAs, investigate their involvement with carboplatin response in OC cell lines and SOC-derived organoids, and search for targets actionable by epigenetic drugs (epi-drugs) that may interfere with SOC response to chemotherapy.

METHODS AND PATIENTS Tumor-derived organoids (TDOs) were established from malignant effusions (pleural effusion and ascites) of 15 patients with SOC undergoing different therapeutic regimens. RNA-Seq data from TDOs, seven normal ovarian tissue samples, and carboplatin-resistant and -sensitive cell lines were obtained and analyzed. The data was compared with external RNA-Seq data from SOC-derived organoids, SOC samples (The Cancer Genome Atlas), and normal ovarian tissue samples (The Genotype Tissue Expression). Public databases were used to filter differentially expressed genes involved in epigenetic regulation and predict interactions between gene products. LncRNAs potentially interacting with the epigenetic machinery were investigated and selected for functional assays using carboplatin-resistant OC cell lines.

RESULTS We identified lncRNAs, including *SNHG12* and *MEG3*, and protein-coding genes involved in epigenetic regulation differentially expressed in the cell lines and TDOs. The abnormal expression of these lncRNAs was confirmed with the external datasets. In resistant cell lines, increased response to carboplatin was observed following *SNHG12* knockdown. Furthermore, after treatment with the epi-drug decitabine, alone or in combination with tazemetostat, the cell lines and one TDO demonstrated enhanced sensitivity to carboplatin, and increased *MEG3* expression in cell lines.

CONCLUSION We identified dysregulated lncRNAs in SOC that can interact with epigenetic effectors and are promising targets for overcoming chemoresistance.

Listas de abreviaturas e siglas

2D	Bidimensional
3D	Tridimensional
ANRASSF1	Antisense noncoding RASSF1
BRCA1	BRCA1 DNA repair-associated
CA125	Cancer antigen 125
CCAT1-L	Colon cancer associated transcript-1- long isoform
CERNA2	Competing endogenous lncrna 2 for microRNA let-7b
ceRNAs	RNAs competidores endógenos
CLIP-seq	Crosslinking Immunoprecipitation and High-throughput Sequencing
CRISPR/Cas	Clustered regulatory interspaced short palindromic repeats/ CRISPR-associated
CTCF	CCCTC-binding factor
DFS	Sobrevivida livre de doença
EOC	Câncer epitelial de ovário
Epi-drugs	Drogas epigenéticas
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FIGO	Federação Internacional de Ginecologia e Obstetrícia
GADD45A	Growth arrest and DNA damage-inducible alpha
GASK1B	Golgi associated kinase 1B
GEO	Gene Expression Omnibus
H19	H19 imprinted maternally expressed transcript
HDAC9	Histone deacetylase 9
HGSOC	High-Grade Serous Ovarian Cancer
HOTAIR	HOX transcript antisense RNA
IL6R	Interleukin 6 receptor
ITGA5	Integrin subunit alpha 5
LGSOC	Low-Grade Serous Ovarian Cancer
lncRNAs	RNAs longos não codificantes
MALAT1	Metastasis associated lung adenocarcinoma transcript 1
MEG3	Maternally expressed 3
miRNAs	MicroRNAs
mRNAs	RNAs mensageiros
MUC16	Mucin 16 cell surface associated
ncRNAs	RNAs não codificantes

NEAT1	Nuclear paraspeckle assembly transcript 1
NF-κB	Nuclear factor kappa B
OC	Câncer de ovário
OS	Sobrevida global
PDX	Xenoinxertos derivados de pacientes
PFI	Sobrevida livre de platina
piRNAs	RNAs que interagem com piwi
PLADE	Platinum sensitivity-related lncRNA from ascites-derived exosomes
PRC2	Polycomb Repressive Complex 2
PVT1	Pvt1 oncogene
RASSF1A	RAS-association domain family member 1A
RNA-seq	Sequenciamento de RNA
SCIRT	Stem cell inhibitory RNA transcript
siRNAs	Pequenos RNAs de interferência
SNHG	Small nucleolar RNA host genes
SNHG12	Small nucleolar RNA host gene 12
snoRNAs	Pequenos RNAs nucleolares
snRNAs	Pequenos RNAs nucleares
SOC	Carcinoma seroso de ovário
SOX4	SRY-box transcription factor 4
TARID	TCF21 antisense RNA inducing promoter demethylation
TCF21	Transcription factor 21
TCGA	The Cancer Genome Atlas
TDG	Thymine DNA glycosylase
TDOs	Organoides derivados de tumor
TET	Ten-eleven translocation
TIMP1	Inibidor da metaloproteinase de matriz-1
tRNAs	RNAs transportadores
TUG1	Taurine up-regulated 1
UKCTOCS	UK Collaborative Clinical Trial of Ovarian Cancer Screening
VEGF	Vascular endothelial growth factor
ZFAS1	ZNFX1 antisense RNA 1

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1. Introdução

1.1. O câncer de ovário

O câncer de ovário (OC, do inglês: *Ovarian Cancer*) é um problema de saúde global, tendo acumulado 324.603 novos casos e 206.956 mortes em 2022 ¹. No Brasil, ocorreram 3.920 óbitos por OC em 2020 e são esperados 7.310 novos casos para cada ano do triênio 2023-2025 ². O OC ocupa a oitava posição dentre os tipos de câncer mais incidentes entre mulheres, excluindo-se o câncer de pele não melanoma, no Brasil e no mundo ^{2,3}. Apesar de não ser o tipo de neoplasia ginecológica mais comum, o OC é frequentemente agravado pela associação com a presença de recidiva e quimiorresistência ⁴. O OC é diagnosticado em estágios avançados em aproximadamente 75% dos casos, em parte por ser assintomático ou apresentar sintomas inespecíficos no início da doença, assim como pela propensão a se disseminar na cavidade peritoneal ⁵⁻⁷. Além disso, não há um método de *screening* efetivo que possa ser amplamente adotado para o diagnóstico precoce. Neste contexto, é possível compreender por que o OC corresponde ao segundo tipo de câncer ginecológico mais letal mundialmente, superado apenas pelo câncer de colo uterino ³.

O risco de desenvolver OC é aumentado por fatores genéticos e hereditários conhecidos, como histórico familiar de câncer de mama ou de ovário e mutações germinativas nos genes *BRCA1* (*BRCA1 DNA repair-associated*) e *BRCA2* (3,6% e 3,3% dos casos, respectivamente) ou em outros genes envolvidos na via de reparo por recombinação homóloga ⁸. Entretanto, outros fatores não-hereditários podem influenciar as diferenças geográficas, étnicas e socioeconômicas observadas na incidência da doença ⁸. A taxa de incidência no Reino Unido é uma das maiores registradas ¹. Na Inglaterra, aproximadamente 35% das mulheres diagnosticadas sobrevivem 10 anos ou mais ⁹. No entanto, quase metade dos casos de OC é detectada nos estágios III e IV ¹⁰. No estágio mais avançado, a taxa de sobrevida em cinco anos

cai para 16%, em oposição à taxa de 94,5% quando o diagnóstico é feito no estágio I ¹⁰. O estudo clínico UKCTOCS (do inglês: *UK Collaborative Clinical Trial of Ovarian Cancer Screening*), o mais recente e abrangente até o momento, incluiu 202.638 mulheres britânicas não pertencentes ao grupo com risco elevado para desenvolver OC familiar ¹¹. Entre essas participantes, foi avaliada a influência de duas estratégias de *screening* anual sobre a mortalidade decorrente de OC, tubas uterinas ou peritônio. As lesões malignas nesses três sítios primários pouco diferem umas das outras quanto à apresentação e ao tratamento e são agrupadas sob os mesmos critérios de classificação ¹¹. Um grupo de mulheres foi submetido apenas à ultrassonografia transvaginal, enquanto no outro foi aplicada uma abordagem multimodal, constituída por acompanhamento de variações nos níveis séricos do biomarcador CA125 (do inglês: *cancer antigen 125*) ao longo do tempo e por exames de ultrassom como segunda linha. Ao mesmo tempo em que uma maior quantidade de casos foi diagnosticada no estágio I em ambos os grupos comparativamente ao grupo controle, não foi verificada redução significativa na mortalidade ¹¹. O marcador sérico CA125, codificado pelo gene *MUC16* (*mucin 16, cell surface associated*), é utilizado para monitorar as mulheres do grupo de alto risco de OC, assim como a resposta à quimioterapia e o surgimento de recidiva. 80% dos tumores e 50% das pacientes em estágios iniciais do OC expressam níveis aumentados dessa glicoproteína, o que demonstra que sua especificidade e sensibilidade limitam seu uso como biomarcador individual ¹². Dessa forma, é essencial que sejam identificados novos marcadores que complementem as estratégias existentes, possibilitando que o OC seja detectado precocemente, contribuindo para a melhora no prognóstico das pacientes. Contudo, essa busca é dificultada por se tratar de uma doença complexa, caracterizada por heterogeneidade molecular, histológica e clínica ¹³.

O OC não é considerado uma entidade única e pode ser classificado em diferentes subtipos, de acordo com a origem celular (epitélio, estroma do cordão sexual, células germinativas ou tipos celulares mistos) e aspectos histológicos ⁸. O câncer epitelial de ovário

(EOC, do inglês: *Epithelial Ovarian Cancer*), corresponde a cerca de 90% dos tumores malignos desse órgão e compreende vários subtipos: seroso de baixo (LGSOC, do inglês: *Low-Grade Serous Ovarian Cancer*) e de alto grau (HGSOC, do inglês: *High-Grade Serous Ovarian Cancer*), endometriode, carcinoma de células-claras e mucinoso ⁸. Dentre esses, o HGSOC é o mais comumente diagnosticado e responde por 70% a 80% das mortes decorrentes de OC ⁸.

O HGSOC é tipicamente responsivo à quimioterapia ao diagnóstico. Os principais quimioterápicos utilizados em casos de OC recém-diagnosticados são os análogos de platina, como a cisplatina ou a carboplatina, combinados com um taxano, como o paclitaxel ou o docetaxel ^{14,15}. Entretanto, estima-se que 80% das pacientes diagnosticadas em estágio avançado da doença sofrerão recidiva e o aspecto mais difícil no tratamento dessas mulheres é o desenvolvimento de resistência à platina ⁴. A classificação atual dos tumores recidivados quanto à sensibilidade a agentes de platina é baseada na sobrevida livre de platina (PFI, do inglês: *Platinum-Free Interval*), isto é, no tempo transcorrido entre a administração da última dose do quimioterápico e a evidência clínica de progressão tumoral. Contudo, esse parâmetro é muito simplista e limitado, não refletindo todo o espectro de respostas ao tratamento em mulheres com EOC ¹⁶. Portanto, novos estudos são necessários a fim de aprimorar a estratificação das pacientes de acordo com o risco para a progressão da doença e com a resposta ao tratamento, por meio da incorporação de novos parâmetros tumorais e imunológicos, assim como de marcadores moleculares, como os RNAs longos não codificantes (lncRNAs) ¹⁷.

1.2. Os RNAs longos não codificantes e seu envolvimento no OC

Estima-se que aproximadamente 80% do genoma humano encontra-se bioquimicamente associado à transcrição, enquanto 62% são transcritos em RNAs de vários tipos e apenas 1,2% está associado a éxons codificadores de proteínas ¹⁸. Isso sugere que a maior parte das sequências de DNA está apta a ser transcrita, mas não a codificar proteínas,

gerando os chamados RNAs não codificantes (ncRNAs). Essa informação alterou a percepção sobre essa classe de RNAs, agora reconhecidos como reguladores cruciais em programas fisiológicos que ocorrem durante o desenvolvimento e em doenças humanas, incluindo o câncer.

Os ncRNAs são capazes de desempenhar funções regulatórias e estruturais e podem ser divididos em duas categorias principais, de acordo com o tamanho das moléculas não codificantes: RNAs longos e RNAs curtos (Figura 1) ¹⁹. Os representantes mais conhecidos dessa última classe são os microRNAs (miRNAs), responsáveis por regular em nível pós-transcricional a expressão de aproximadamente 50% dos produtos gênicos ²⁰. Os lncRNAs constituem um grupo de transcritos com mais de 200 nucleotídeos que geralmente não são traduzidos em proteínas, embora alguns deles sejam capazes de codificar pequenos peptídeos ²¹. Com base na sua posição no genoma e relativamente aos genes codificadores de proteínas, os lncRNAs podem ainda ser classificados como intergênicos, *sense*, *antisense*, bidirecionais ou intrônicos. Em comparação com os RNAs mensageiros (mRNAs), que são traduzidos em proteínas, muitos dos lncRNAs ficam restritos ao núcleo, enquanto outros se concentram no citoplasma ²². São amplamente expressos nos tecidos humanos e, embora menos abundantes, apresentam maior especificidade tecidual em comparação com os transcritos codificadores de proteínas, característica que reforça o potencial dos lncRNAs como biomarcadores ^{23,24}.

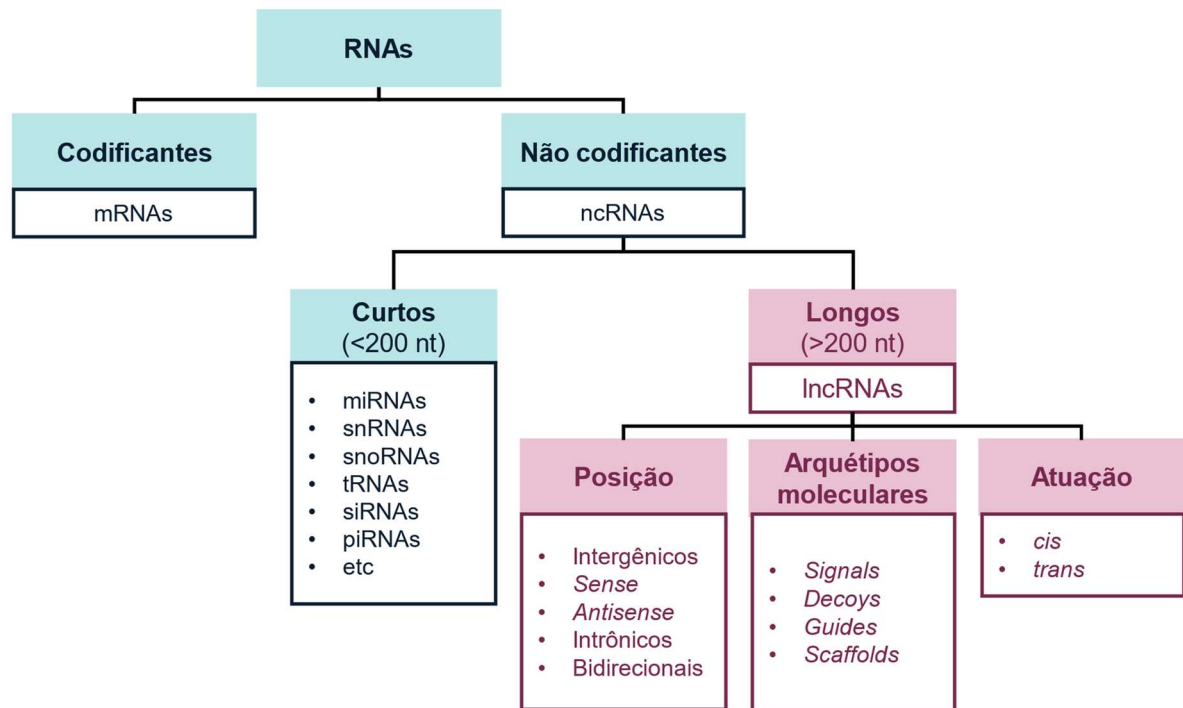


Figura 1. Tipos de moléculas de RNA. Os RNAs são classificados quanto à sua capacidade de codificar proteínas, ao seu comprimento e, no caso dos RNAs longos não codificantes (lncRNAs), podem ser categorizados de acordo com a sua posição em relação aos genes codificadores de proteínas, o seu arquétipo molecular e o seu alcance de atuação. mRNAs: RNAs mensageiros; ncRNAs: RNAs não codificantes; miRNAs: microRNAs; snRNAs: pequenos RNAs nucleares; snoRNAs: pequenos RNAs nucleolares; tRNAs: RNAs transportadores; siRNAs: pequenos RNAs de interferência; piRNAs: RNAs que interagem com *piwi*.

Considerando suas atividades em nível molecular, os lncRNAs podem agir como *signals*, *decoys*, *guides* ou *scaffolds*. No primeiro arquétipo, o padrão de ativação ou repressão da cromatina pode ser inferido a partir da expressão dos lncRNAs associados a ela, sinalizando eventos determinantes para a regulação gênica. Os lncRNAs que atuam como *decoys* se ligam a proteínas-alvo, como fatores de transcrição e modificadores da cromatina, ou a pequenos RNAs regulatórios e os atraem para longe de seus sítios de destino, modulando negativamente a atividade de tais efetores ²⁵. Existe uma subclasse de lncRNAs, os competidores endógenos (ceRNAs, do inglês: *competing endogenous RNAs*), que atuam como “esponjas” para os miRNAs. Assim, os ceRNAs sequestram um ou mais desses transcritos e permitem que seus respectivos alvos sejam traduzidos ²⁶. Os lncRNAs que atuam como *guides* se ligam a proteínas e então direcionam os complexos formados aos seus alvos específicos em *cis*, ou seja, em genes

próximos ao sítio de transcrição dos lncRNAs, ou em *trans*, isto é, em genes distantes, até mesmo em outros cromossomos. Os lncRNAs também podem servir como *scaffolds* sobre os quais importantes componentes moleculares, com atividades repressoras ou ativadoras da transcrição, são montados. Portanto, esses lncRNAs se ligam simultaneamente a múltiplas moléculas efetoras, por meio de diferentes domínios, e, assim, as combinam no tempo e espaço²⁵.

Essa variedade de domínios estruturais contribui para a versatilidade funcional dos lncRNAs, permitindo sua interação com moléculas de DNA, RNA e proteínas e sua participação no ajuste fino da expressão gênica em diversos níveis de regulação, incluindo o controle epigenético da transcrição²⁷. A atuação dos lncRNAs em vias epigenéticas pode afetar a arquitetura da cromatina, a modificação de histonas em *cis* ou em *trans* e a metilação do DNA (Figura 2)²⁸. A influência dos lncRNAs na arquitetura da cromatina pode ocorrer por meio da ligação com proteínas que atuam na formação de *loops* e facilitar a interação entre elementos regulatórios e unidades de transcrição próximas ou situadas em outros cromossomos. Como exemplo, o lncRNA *CCAT1-L* (*colon cancer associated transcript-1 - long isoform*) interage diretamente com a proteína CTCF (*CCCTC-binding factor*), mediando a formação de *loops* na cromatina que aproximam o promotor do gene *MYC* de seus acentuadores, favorecendo a expressão desse oncogene²⁹. Os lncRNAs também podem interagir com complexos que adicionam ou removem modificações pós-traducionais em aminoácidos localizados nas caudas amino-terminais das histonas. *ANRASSF1* (*antisense noncoding RASSF1*) é um lncRNA *antisense* que interage com o DNA na região promotora do gene supressor tumoral *RASSF1A* (*RAS-association domain family member 1A*), formando um híbrido de RNA/DNA, o qual recruta o complexo PRC2 (*Polycomb Repressive Complex 2*)³⁰. Por meio da sua subunidade catalítica EZH2 (*enhancer of zeste 2 polycomb repressive complex 2 subunit*), esse complexo introduz modificações repressivas, equivalentes à metilação da lisina 27 da histona 3

(H3K27me3), promovendo o silenciamento transcricional de *RASSF1A* ^{30,31}. Os lncRNAs ainda são capazes de interferir na metilação do DNA em células humanas ao recrutar ou inibir a ação de DNA metiltransferases e desmetilases. Um exemplo é o lncRNA *antisense TARID (TCF21 antisense RNA inducing promoter demethylation)*, que medeia a desmetilação do supressor tumoral *TCF21 (transcription factor 21)*, codificado pela fita *sense* do DNA ³². *TARID* se liga ao promotor do seu gene-alvo e recruta a proteína GADD45A (*growth arrest and DNA damage-inducible alpha*), que, por sua vez, atrai a maquinaria de desmetilação do DNA para ativar a transcrição de *TCF21* ³². As proteínas da família TET (*ten-eleven translocation*), em especial, são reconhecidas pelo seu papel como desmetilases em mamíferos; elas oxidam a 5-metilcitosina (5mC) em 5-hidroximetilcitosina (5hmC), que é oxidada em 5-formilcitosina (5fC) ou 5-carboxilcitosina (5caC). A maquinaria de reparo por excisão de base promove a substituição de 5fC e 5caC por citosinas não modificadas por meio da excisão desses resíduos pela proteína TDG (*thymine DNA glycosylase*) e do reparo do sítio apirimidínico resultante ³³.

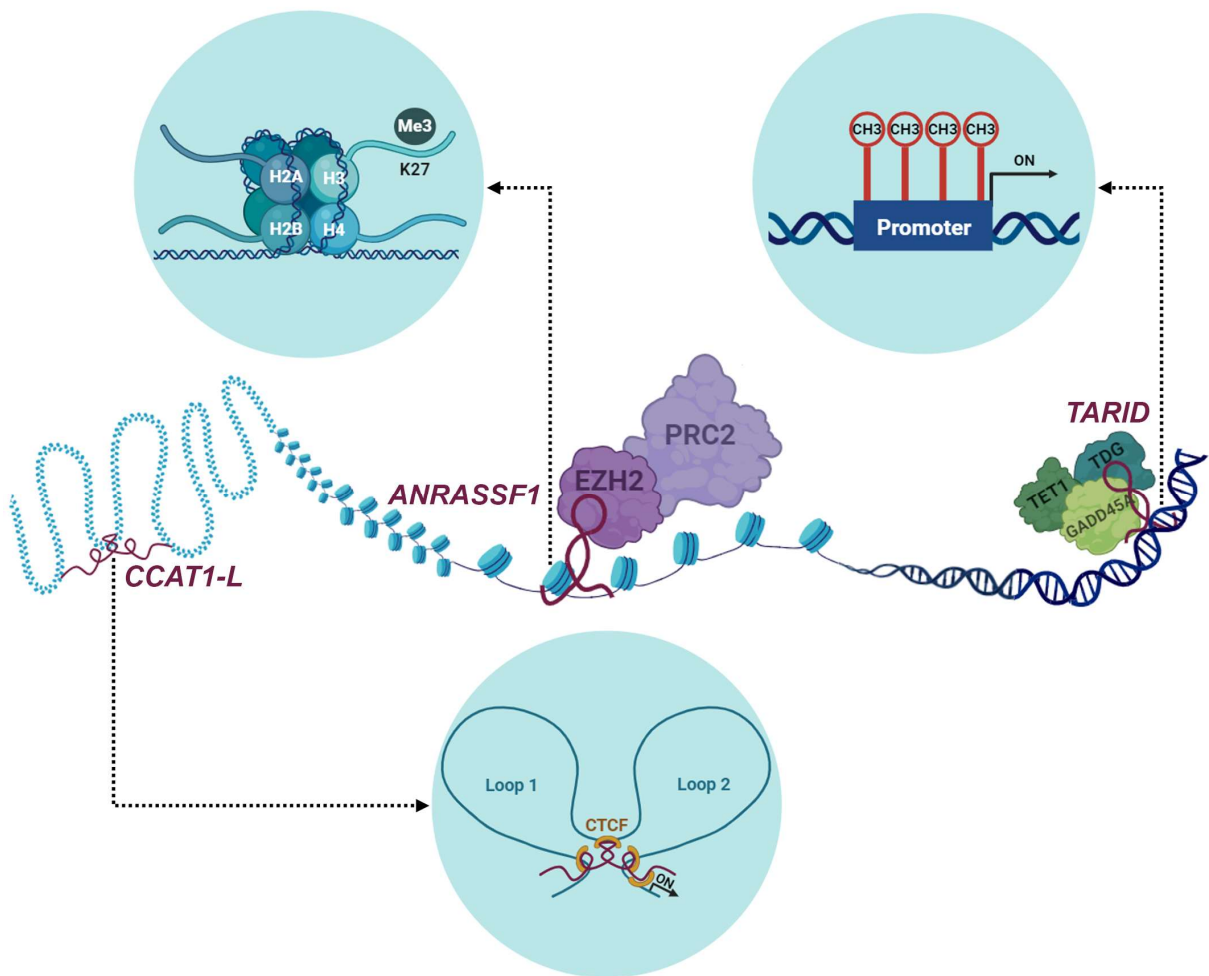


Figura 2. Exemplos do envolvimento dos RNAs longos não codificantes (lncRNAs) no controle epigenético da expressão gênica. Os lncRNAs *CCAT1-L* (*colon cancer associated transcript-1 - long isoform*), *ANRASSF1* (*antisense noncoding RASSF1*) e *TARID* (*TCF21 antisense RNA inducing promoter demethylation*) interagem com efetores epigenéticos que promovem a formação de *loops* na cromatina, a adição de modificações repressivas em histonas e a desmetilação do DNA, respectivamente, modulando a acessibilidade da cromatina à maquinaria de transcrição. Figura criada com BioRender.com.

Um mesmo lncRNA pode exercer sua atividade por meio de diversos mecanismos moleculares. *HOTAIR* (*HOX transcript antisense RNA*) é um dos lncRNAs mais amplamente estudados e sua expressão anormal já foi relatada em mais de 20 tipos de câncer, incluindo tumores de mama e de ovário³⁴. Esse lncRNA pode atuar como *scaffold* e *guide* para complexos modificadores da cromatina, assim como competidor endógeno para diversos miRNAs em vias relacionadas ao câncer, entre outras atividades. *HOTAIR* desempenha funções oncogênicas e sua expressão está comumente aumentada no câncer, mas, ao contrário dos oncogenes clássicos,

raramente apresenta variação do número de cópias, de modo que sua desregulação ocorre em nível transcricional ³⁵. No câncer de mama, seus níveis aumentados foram associados com maior capacidade de invasão e de metástase, enquanto a sua depleção inibiu a invasão ³⁴. No EOC, *HOTAIR* está envolvido na proliferação por meio do controle do ciclo celular e apoptose, além de promover a invasão e a metástase, constituindo um importante fator prognóstico independente ^{36,37}.

Evidências recentes indicam que alguns lncRNAs estão implicados na origem e progressão do EOC. Wang *et al.* (2016) descreveram a expressão diferencial de 663 lncRNAs no EOC comparado a tecidos normais e lesões benignas ³⁸. Os autores verificaram que os níveis de expressão do transcrito *antisense RP11-597D13.9* estavam positivamente correlacionados com *GASK1B* (*golgi associated kinase 1B*), um gene codificador mapeado próximo conhecido por participar da progressão tumoral ³⁸. Outros 62 lncRNAs tiveram sua expressão diferencial detectada no EOC resistente à cisplatina quando comparado a controles sensíveis à quimioterapia ³⁹. Em outro estudo, os autores traçaram um amplo panorama de alterações no transcriptoma do HGSOC em comparação com o tecido normal das tubas uterinas ⁴⁰. Os níveis de cinco lncRNAs (*GTSE1-AS1*, *FAS-AS1*, *AK130076*, *RP11-199F11.2* e *AC093818.1*) exibiram associação significativa com o estadiamento pelo sistema da Federação Internacional de Ginecologia e Obstetrícia (FIGO, do francês: *Fédération Internationale de Gynécologie et d'Obstétrique*) e com a ocorrência de metástase em linfonodos.

A partir da análise sistemática do perfil de expressão de lncRNAs e de mRNAs no *The Cancer Genome Atlas* (TCGA), Fang *et al.* (2018) identificaram uma rede de interações entre lncRNAs e mRNAs relacionada com a resistência à platina no HGSOC ⁴¹. Nessa rede, 35 lncRNAs e 270 mRNAs apresentaram 124 relações de co-expressão significativas. O estudo revelou que muitos dos genes envolvidos na quimiorresistência estão implicados em vias metabólicas e que o lncRNA *SCIRT* (*stem cell inhibitory RNA transcript*) pode atuar por meio

da desregulação de miRNAs, desempenhando o papel de competidor endógeno. Dados de sequenciamento de RNA (RNA-Seq) do TCGA resultaram em outra assinatura (*LINC01673*, *MIR137HG*, *PLCH1-AS1*, *AC009988.1*, *C20orf78*, *AC003659.1*, *AL162713.1* e *AC099542.1*), a qual foi capaz de prever a sensibilidade à quimioterapia baseada em platina em pacientes com SOC ⁴².

O perfil de expressão dos lncRNAs em pacientes com EOC também foi analisado a partir de conjuntos de dados disponíveis publicamente no *Gene Expression Omnibus* (GEO). Utilizando dados de pacientes com HGSOC, Song *et al.* (2018) identificaram uma assinatura composta por sete lncRNAs (*RFX5-AS1*, *ZBED3-AS1*, *RP11-439E19.10*, *RP11-348N5.7*, *GRASLND*, *GAS5* e *F11-AS1*), enquanto Liu *et al.* (2017) identificaram uma assinatura com oito lncRNAs (*ZFAS1*, *RP5-1061H20.5*, *RP11-489O18.1*, *LINC02352*, *LINC01514*, *TUG1*, *RP11-1361I4.5* e *CTD-2555A7.3*) ^{43,44}. Os dois estudos não apresentaram sobreposição entre os lncRNA identificados, mas ambos descreveram o potencial destas assinaturas na predição de resistência à platina. Wang *et al.* (2017), por sua vez, reportaram uma assinatura com outros sete lncRNAs (*XR_948297*, *XR_947831*, *XR_938728*, *XR_938392*, *NR_103801*, *NR_073113* e *NR_036503*), a qual foi significativamente correlacionada com a resposta desfavorável à quimioterapia com paclitaxel nas pacientes com EOC ⁴⁵. Xu *et al.* (2019) descreveram uma assinatura com 10 lncRNAs obtida por meio da análise de dados de 343 casos do TCGA ⁴⁶. As pacientes com HGSOC estratificadas no grupo de alto risco, de acordo com o preditor linear dessa assinatura prognóstica, experienciaram sobrevida livre de doença (DFS, do inglês: *Disease-free Survival*) e sobrevida global (OS, do inglês: *Overall Survival*) menores, além de serem potencialmente resistentes à terapia baseada em platina.

Mais de 20 lncRNAs foram implicados na resistência à terapia baseada em platina no OC, mas poucos tiveram suas funções subjacentes elucidadas ⁴⁷. A desregulação de alguns desses transcritos, como *H19* (*H19 imprinted maternally expressed transcript*) e *HOTAIR*, foi

relacionada à menor sobrevida em pacientes tratadas com análogos de platina. Em uma meta-análise recente, a expressão aumentada de *SNHG12* (*small nucleolar RNA host gene 12*) foi correlacionada com pior OS em pacientes com câncer ⁴⁸. Em um estudo prévio do grupo, *SNHG12* foi selecionado para ensaios funcionais a partir de um conjunto de 11 lncRNAs diferencialmente expressos e metilados em linhagens de OC sensíveis e resistentes à carboplatina ⁴⁹. O silenciamento desse lncRNA induziu a resposta ao quimioterápico em linhagens intrinsecamente resistentes, sugerindo seu potencial como alvo terapêutico. Outros lncRNAs, como *MALAT1* (*metastasis associated lung adenocarcinoma transcript 1*), *CERNA2* (*competing endogenous lncRNA 2 for microRNA let-7b*), *PVT1* (*Pvt1 oncogene*), *NEAT1* (*nuclear paraspeckle assembly transcript 1*) e *MEG3* (*maternally expressed 3*), foram igualmente implicados no EOC, sendo que as expressões aumentadas de *PVT1* e de *HOTAIR* foram associadas à resistência à cisplatina em linhagens celulares derivadas de OC por meio da regulação de fatores apoptóticos e da ativação da via do NF- κ B (*nuclear factor kappa B*), respectivamente ^{50–52}. Por outro lado, o aumento dos níveis de *PVT1* mediante a quimioterapia baseada em platina também foi relacionado com a indução da proteína p53 e da expressão do inibidor tecidual da metaloproteinase de matriz-1 (TIMP1), simultaneamente à diminuição da proliferação celular ⁵³. Uma meta-análise contendo diversos *datasets* do GEO confirmou a participação de *MALAT1* na resistência à quimioterapia convencional utilizada no EOC e revelou a influência de outros lncRNAs não associados à quimiorresistência no EOC (*LINC00667*, *NRSN2-AS1* e *RAD51-AS1*) ou associados a outros tipos de câncer (*DLEU2*, *LINC00667* e *PRKCQ-AS1*) ⁵⁴. A expressão reduzida de *MEG3* foi associada a menor OS e estadiamento FIGO mais avançado em pacientes com OC ^{55,56}. Um estudo recente sugeriu que *MEG3*, *PVT1* e *TUG1* (*taurine up-regulated 1*) poderiam compor uma assinatura preditora da resistência à cisplatina no OC ⁵⁷.

Mais um exemplo a ser citado é a descoberta de que o silenciamento da isoforma 2 de *NEAT1* sensibiliza as células tumorais à quimioterapia ⁵⁸. Outro lncRNA, *ZFAS1* (*ZNFx1 antisense RNA 1*), além de ter seus níveis elevados correlacionados com a menor sensibilidade à cisplatina compondo uma das assinaturas com potencial preditivo no OC citadas anteriormente, apresenta expressão presumivelmente controlada por esse quimioterápico ⁴⁴. O silenciamento de *HOTAIR* foi capaz de suprimir a proliferação celular, reduzindo a invasão e restaurando a sensibilidade em células resistentes à cisplatina SKOV-3CDDP/R derivadas de carcinoma seroso de ovário (SOC, do inglês: *serous ovarian carcinoma*) ⁵⁹. Recentemente, Liu *et al.* (2024) comprovaram que a expressão aumentada de um novo lncRNA, *PLADE* (*platinum sensitivity-related lncRNA from ascites-derived exosomes*), contribuiu para a resposta favorável à cisplatina ⁶⁰. Esse lncRNA apresentou níveis maiores em exossomos de ascite e em tecidos tumorais de pacientes com HGSOC responsivas ao quimioterápico. Experimentos funcionais revelaram que *PLADE* promove a sensibilidade à cisplatina suprimindo a proliferação, a migração e a invasão e induzindo a apoptose *in vitro* e *in vivo*. Em conjunto, todas essas evidências sinalizam o potencial dos lncRNAs como biomarcadores prognósticos e preditivos, assim como alvos para modular a agressividade do OC e sua resposta à terapia baseada em platina.

1.3. O modelo experimental: organoides derivados de tumor

Apesar dessas descobertas promissoras e do grande número de lncRNAs identificados no genoma humano, poucos foram bem caracterizados e explorados. Há a necessidade de compreensão dos mecanismos que influenciam a patogênese do SOC, sobretudo daqueles relacionados à resistência à platina ¹⁷. Portanto, o delineamento de abordagens que auxiliem na elucidação do papel dos lncRNAs na resistência à quimioterapia no SOC pode ajudar a compreender tais mecanismos. Nesse aspecto, a cultura celular tridimensional (3D) tem sido

relatada como um modelo fiel e custo-efetivo para estudar o SOC quando comparada aos modelos de cultura bidimensional (2D) ou xenoenxertos derivados de pacientes (PDX, do inglês: *Patient-Derived Xenografts*)⁶¹. Os organoides derivados de tumor (TDOs, do inglês: *Tumor-Derived Organoids*) são estruturas 3D cultivadas *in vitro* com capacidade de recapitular a complexidade molecular e a resistência à quimioterapia de diferentes tipos de tumor, incluindo o EOC⁶². Sua conceitualização frequentemente se confunde com a de outro modelo 3D: os esferoides. Estes, porém, geralmente são apenas agregados celulares desprovidos de componentes de matriz extracelular, não reproduzem a arquitetura do tecido de origem nem apresentam diversidade de células, constituindo estruturas mais simples e homogêneas do que os organoides⁶³. À medida em que crescem em volume, os esferoides desenvolvem um centro necrótico resultante do comprometimento na difusão de nutrientes e oxigênio através das camadas de células⁶⁴. Desse modo, os TDOs são mais adequados para testes com quimioterápicos, por exemplo, uma vez que as drogas que promovem morte celular por meio da formação de espécies reativas de oxigênio (e.g. análogos de platina) têm sua eficácia prejudicada na região hipóxica no centro dos esferoides⁶⁴. Adicionalmente, as drogas que são mais eficazes em células com alta taxa proliferativa (e.g. paclitaxel) apresentam efeito menor nas camadas mais internas dos esferoides, compostas por células senescentes e necróticas⁶⁴. Os TDOs podem ser gerados a partir de tecidos sólidos obtidos por ressecção cirúrgica e de agregados de células presentes em amostras de fluidos de pacientes com câncer, incluindo ascites, e utilizados como plataforma para o *screening* personalizado de drogas⁶⁵.

O OC é o sexto tipo de câncer associado com a ocorrência de efusão pleural, enquanto a ascite é observada em mais de um terço das pacientes^{66,67}. No OC, o acúmulo de ascite ocorre se a drenagem linfática do abdome for obstruída por células tumorais disseminadas na cavidade peritoneal ou se a produção de fluido aumentar, o que é estimulado pelo aumento dos níveis de VEGF (*vascular endothelial growth factor*) e consequente aumento da permeabilidade capilar

⁶⁸. A incidência e o volume de ascite são maiores em estágios avançados do OC e o aumento do volume foi associado com pior prognóstico ⁶⁹. A efusão pleural no OC presumivelmente resulta da invasão de estruturas contíguas, como o diafragma, ou da migração de células tumorais através da comunicação pleuroperitoneal ⁷⁰. Além disso, a disseminação pleural pode ocorrer pela via hematogênica. A ocorrência de efusão pleural também foi relacionada com prognóstico desfavorável no OC ⁷¹. Ambos os tipos de efusões malignas constituem um material promissor para a produção *in vitro* de TDOs e para estratégias de biópsia líquida pois contêm células tumorais e não-tumorais, como fibroblastos e células inflamatórias, além de *cell-free* DNA e RNA e moléculas sinalizadoras ^{68,72}. Outra vantagem em gerar organoides a partir de amostras de lesões metastáticas, como as efusões malignas, é a menor chance de contaminação do material com células epiteliais normais ⁶⁵. A ascite derivada de OC contém uma grande quantidade de agregados de células estruturalmente organizados em esferoides ⁷³. Fibroblastos associados ao câncer recrutam e promovem a expressão da integrina alpha 5 (ITGA5) nas células de OC presentes na ascite, compondo esferoides invasivos que são fundamentais para a disseminação intraperitoneal do tumor ⁷³. As células epiteliais derivadas de OC dos esferoides apresentam menor capacidade proliferativa, mas maior potencial tumorigênico em comparação com células tumorais aderentes ⁷³.

Um estudo que comparou organoides derivados de SOC e células primárias cultivadas em 2D relatou que as respostas a drogas foram mais diversas e fortemente correlacionadas com alterações genômicas nos organoides em comparação à cultura em monocamada ⁷⁴. Os resultados do sequenciamento de genoma completo das amostras das pacientes e do tratamento dos organoides demonstraram associações entre a deficiência no reparo do DNA e a sensibilidade a drogas que não haviam sido detectadas nas culturas 2D, como é o caso da resposta ao agente desmetilante decitabina. Maenhoudt *et al.* (2017) produziram organoides a partir de biópsias de EOC, majoritariamente SOC, e demonstraram que esses modelos são

adequados para a triagem de drogas de forma personalizada ⁷⁵. As curvas dose-resposta exibiram os diferentes níveis de sensibilidade dos organoides aos quimioterápicos testados (carboplatina, paclitaxel, doxorubicina e gemcitabina), sugerindo que as respostas apresentadas *in vitro* são paciente-dependentes. Além disso, os organoides reproduziam outros aspectos celulares e moleculares do fenótipo tumoral, incluindo a expressão de *MUC16*. Um estudo mais recente demonstrou que a sensibilidade dos organoides derivados de HGSOC às drogas comumente usadas no tratamento desse tumor se correlacionou com a resposta clínica das pacientes, mas também foi dependente das condições de cultivo ⁷⁶. A resposta dos organoides submetidos ao tratamento em meio de cultura que mimetizava o plasma humano apresentaram correlações mais fortes com os níveis de CA125 das pacientes após a quimioterapia. Ainda não existe uma padronização em relação à formulação do meio de cultura, aos procedimentos para estabelecer e manter os TDOs e aos critérios para avaliar sua resposta ao teste com drogas ⁷⁷. Isso ajuda a explicar as diferenças nas taxas de sucesso ao gerar organoides derivados de OC observadas em diferentes estudos, variando de 30% a quase 100% ⁷⁶⁻⁷⁹.

Os TDOs são ferramentas úteis para avaliar *ex vivo* a resposta dos pacientes oncológicos a diferentes regimes terapêuticos. Sua implementação deve ser considerada com cautela dada a complexidade do modelo e a ausência de procedimentos padronizados para sua obtenção. No presente estudo, TDOs gerados a partir de amostras de ascite e efusão pleural de pacientes com carcinoma seroso de ovário foram o modelo selecionado para capturar o perfil de expressão de lncRNAs. A partir de dados de RNA-Seq obtidos de linhagens celulares sensíveis e resistentes à carboplatina e de organoides derivados de SOC, este estudo selecionou lncRNAs desregulados possivelmente envolvidos na resistência ao tratamento. Este estudo permitiu identificar lncRNAs que interagem com a maquinaria epigenética, o que pode contribuir para o delineamento de estratégias que utilizem a mobilização do controle epigenético da expressão

gênica para tornar o tratamento com os quimioterápicos convencionais mais eficaz.

2. *Justificativa e Hipótese*

O câncer de ovário ocupa a segunda posição entre as neoplasias ginecológicas malignas mais letais ³. Seu subtipo histológico mais comum, o HGSOC, é responsável por 70% a 80% das mortes pela doença ⁸. Muitas das pacientes recebem o diagnóstico tardiamente, principalmente devido à falta de métodos de *screening* efetivos, e sofrem com tumores recidivados que são refratários à quimioterapia com análogos de platina. Esses agentes alquilantes constituem a abordagem de escolha para tratar tumores recém-diagnosticados, sendo que a cisplatina e a carboplatina são dois dos seus principais representantes ⁴. Nos casos de quimiorresistência, a avaliação da resposta ao tratamento e a escolha de novas estratégias terapêuticas é limitada pela ausência de marcadores preditivos que possibilitem a estratificação adequada das pacientes em grupos de risco ¹⁶. A expressão alterada de alguns lncRNAs foi associada à menor sensibilidade à cisplatina em pacientes com câncer de ovário em diversos estudos; portanto, representam alvos promissores para investigações mais aprofundadas, a fim de esclarecer suas funções na resposta ao tratamento quimioterápico ⁴⁷.

A hipótese deste estudo é que lncRNAs com expressão alterada no SOC interferem na resposta à carboplatina e podem contribuir para o delineamento de terapias mais eficazes para este tipo tumoral.

3. *Objetivos*

3.1. Objetivo geral

Caracterizar a expressão de lncRNAs em organoides derivados de carcinomas serosos de ovário e seu envolvimento na resistência à carboplatina.

3.2. Objetivos específicos

3.2.1. Capítulo 1

- Produzir um guia baseado em bancos de dados públicos que permitam a anotação de lncRNAs e a sua caracterização;
- Utilizar bancos de dados para selecionar lncRNAs alterados no câncer de ovário e associados a mecanismos epigenéticos e comparar com os achados do presente estudo;

3.2.2. Capítulo 2

- Identificar lncRNAs diferencialmente metilados e expressos em linhagens celulares derivadas de câncer de ovário resistentes à carboplatina;
- Avaliar a sensibilidade à carboplatina em linhagens celulares derivadas de câncer de ovário após o silenciamento do lncRNA *SNGH12*, potencialmente envolvido na quimiorresistência;

3.2.3. Capítulo 3

- Cultivar em 3D células obtidas de efusões malignas de carcinomas serosos de ovário para obter organoides derivados de tumor;
- Utilizar RNA-Seq para identificar lncRNAs e componentes da maquinaria epigenética diferencialmente expressos em organoides derivados de carcinomas

serosos de ovário;

- Avaliar a sensibilidade à carboplatina e a expressão do lncRNA *MEG3*, implicado na regulação epigenética, em linhagens celulares e organoides derivados de câncer de ovário após o tratamento com drogas epigenéticas (*epi-drugs*, do inglês: *epigenetic drugs*).

4. Capítulo 1

Este capítulo refere-se ao artigo publicado no periódico *Cancers* (ISSN 2072-6694):

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O artigo de revisão contemplado neste capítulo foi organizado com base em bancos de dados públicos com o objetivo de auxiliar a comunidade científica, incluindo nosso grupo de pesquisa, na seleção da ferramenta mais adequada para explorar os achados relacionados aos lncRNAs. Apresentamos um conjunto abrangente de bancos de dados que incluem informações sobre a anotação e a função dos lncRNAs, assim como sua associação com doenças. Além disso, essas ferramentas incluem evidências da literatura e predições computacionais de forma a dar suporte para a seleção de transcritos candidatos para estratégias experimentais alvo-específicas. Desde esta publicação, vários bancos de dados foram atualizados e outros, desenvolvidos. O banco de dados LncBook (<https://ngdc.cncb.ac.cn/lncbook>), o mais abrangente em termos de número de lncRNAs humanos anotados à época da publicação, está atualmente em sua segunda versão e expandiu sua coleção de transcritos de 270.044 para 323.950⁸⁰. Contudo, a quantidade de genes que codificam lncRNAs diminuiu nessa nova versão, representando um esforço para remover pseudogenes, RNAs pequenos, precursores de miRNAs e transcritos sem informação sobre sua fita de origem a fim de melhorar a qualidade da anotação. De modo semelhante, outros bancos de dados tiveram novas versões lançadas, incluindo as ferramentas NPInter (versão 5.0; <http://bigdata.ibp.ac.cn/npinter5>), especializada em interações entre lncRNAs e outras biomoléculas, e LncRNADisease (versão 3.0;

<http://www.rnanut.net/lncrnadisease>), dedicada a catalogar evidências de associação entre lncRNAs e doenças. Esses foram alguns dos bancos de dados empregados para explorar os resultados do estudo abordado no capítulo 2.

Review

The Interplay between Long Noncoding RNAs and Proteins of the Epigenetic Machinery in Ovarian Cancer

Naiade Calanca ¹, Cecilie Abildgaard ^{2,3}, Cláudia Aparecida Rainho ¹ and Silvia Regina Rogatto ^{3,*}

¹ Department of Chemical and Biological Sciences, Institute of Biosciences, São Paulo State University (UNESP), Botucatu 18618-689, Brazil; naiade.calanca@unesp.br (N.C.); claudia.rainho@unesp.br (C.A.R.)

² Department of Oncology, University Hospital of Southern Denmark-Vejle, Institute of Regional Health Research, University of Southern Denmark, 5000 Odense, Denmark; Cecilie.Abildgaard@rsyd.dk

³ Department of Clinical Genetics, University Hospital of Southern Denmark-Vejle, Institute of Regional Health Research, University of Southern Denmark, 5000 Odense, Denmark

* Correspondence: silvia.regina.rogatto@rsyd.dk; Tel.: +45-7940-6669

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Simple Summary: Epithelial ovarian cancer is an aggressive disease associated with relapse, resistance to chemotherapy, and high mortality rates. Recent discoveries have pointed out that long noncoding RNAs (lncRNAs) are potential biomarkers or therapeutic targets in several tumor types. However, fundamental knowledge about their functions and regulation is still lacking. Here, we present the current understanding of the interplay between lncRNAs and the epigenetic machinery influencing ovarian carcinogenesis. We also provide an overview of bioinformatics tools and databases that can be exploited for lncRNAs investigations. Altogether, this information can support the development of clinical initiatives to monitor disease progression or discover new strategies for the therapeutic management of ovarian cancer.

Abstract: Comprehensive large-scale sequencing and bioinformatics analyses have uncovered a myriad of cancer-associated long noncoding RNAs (lncRNAs). Aberrant expression of lncRNAs is associated with epigenetic reprogramming during tumor development and progression, mainly due to their ability to interact with DNA, RNA, or proteins to regulate gene expression. lncRNAs participate in the control of gene expression patterns during development and cell differentiation and can be cell and cancer type specific. In this review, we described the potential of lncRNAs for clinical applications in ovarian cancer (OC). OC is a complex and heterogeneous disease characterized by relapse, chemoresistance, and high mortality rates. Despite advances in diagnosis and treatment, no significant improvements in long-term survival were observed in OC patients. A set of lncRNAs was associated with survival and response to therapy in this malignancy. We manually curated databases and used bioinformatics tools to identify lncRNAs implicated in the epigenetic regulation, along with examples of direct interactions between the lncRNAs and proteins of the epigenetic machinery in OC. The resources and mechanisms presented herein can improve the understanding of OC biology and provide the basis for further investigations regarding the selection of novel biomarkers and therapeutic targets.

Keywords: lncRNAs; epigenetic regulators; transcriptional regulation; ovarian cancer; lncRNA databases

1. Introduction

Despite the advances in diagnosis and treatment in the last decades, ovarian cancer (OC) is still considered a clinical challenge due to the absence of improvements in long-term survival [1,2]. Based on the Global Cancer Observatory (GLOBOCAN) estimates of cancer incidence and mortality, OC is a health problem worldwide, and the second cause of death among gynecological malignancies, after cervical cancer [3]. In 2018, it accounted for 295,414 new cases and 184,799 deaths globally [4]. This disease is considered complex, being characterized by heterogeneity at molecular, histological, and clinical levels [5]. OC is classified into different subtypes according to cell origin (epithelial, sex-cord stromal, germ cell, and mixed-cell type) and histological features [6]. Epithelial ovarian cancer (EOC) is the most common type of malignancy that affects ovaries (approximately 90%). Furthermore, multiple histological subtypes characterize the EOC, including serous, endometrioid, clear cell, mucinous, and malignant Brenner. Of these, high-grade serous ovarian carcinoma (HGSOC) is the most commonly diagnosed type and accounts for 70–80% of deaths from the whole spectrum of OC subtypes [7]. This cancer type is commonly associated with relapse and chemoresistance [8]. OC is frequently diagnosed at advanced stages, partly because these tumors often exhibit nonspecific symptoms at the first stages of the disease, and due to the absence of effective screening strategies for early detection [9,10]. The aggressive nature of the disease, commonly causing transcoelomic dissemination, adds to the poor prognosis of less than 30% 5-year survival for stage III and IV tumors [11,12]. Platinum-based chemotherapy is the primary treatment option, often combined with a taxane-based drug, namely paclitaxel. Several targeted therapies such as bevacizumab, an inhibitor of vascular endothelial growth factor (VEGF) or PARP (Poly-ADP-ribose-polymerase) inhibitors of proteins can be administered first-line or as a second-line treatment for the recurrent disease [13,14]. Although most patients initially respond well to chemotherapy, it is estimated that 80% of OC patients at advanced-stage will experience recurrence of their primary cancer [8].

The improvement in high-throughput technologies, such as tiling arrays and Next Generation Sequencing (NGS), as well as bioinformatics analyses have extended the boundaries of our knowledge about the noncoding sequences in the human genome. Once considered junk DNA, these sequences underwent a paradigm shift after the discovery that the human genome is pervasively transcribed. Although approximately 80% of the human genome is transcribed into RNAs, only a small fraction of them is associated with protein-coding exons [15,16]. This suggests that most DNA sequences can be transcribed but not to encode proteins, generating noncoding RNAs (ncRNAs). This information has altered our perception of this RNA class and led to increased attention to their functional roles as regulators of physiological programs during development and human diseases, including cancer [17]. These ncRNAs are involved in regulatory and structural functions in cells and are usually are classified into two main categories according to the molecule size: long and short noncoding RNAs. Long noncoding RNAs (lncRNAs) are a group of transcripts with more than 200 nucleotides in length and were initially defined by the apparent absence of protein-coding potential [18].

Through direct interactions with DNA, RNA, or proteins, lncRNAs exert regulatory roles over their target genes at transcriptional, post-transcriptional, and epigenetic levels in a broad range of biological processes, including coordination of allelic expression, cell cycle control, stem cell pluripotency maintenance, differentiation, and apoptosis [19,20]. Considering the relevance of these processes in health and disease and the great diversity of lncRNAs encoded by the human genome, it is not surprising that these transcripts have been linked to almost all hallmarks of cancer [21]. Coined by Hanahan and Weinberg, these hallmarks correspond to the traits acquired by cells that allow malignant transformation [22]. The regulation of gene expression mediated by lncRNAs may involve the epigenetic mechanisms by direct interactions with proteins of the epigenetic machinery, including histone modifications and chromatin remodeling [23]. The interplay between differentially expressed lncRNAs, such as *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) and *CDKN2B-AS1* (cyclin-dependent kinase inhibitor 2B antisense RNA 1), and histone-modifying or chromatin-remodeling complexes has been implicated in transcriptional regulation, enabling

progression of different cancer types [24]. A set of the OC-related lncRNAs acts as oncogenes or tumor suppressors, contributing to cellular transformation [25–27].

The aberrant expression levels of these lncRNAs can be detected in tumor tissues and body fluids. Some lncRNAs were found to be widely dysregulated, such as *HOTAIR* (HOX transcript antisense RNA), whereas others are specifically altered in one or a few tumor types [28,29]. In EOC, it was demonstrated that hundreds of lncRNAs are differentially expressed compared to benign and normal control tissues [30]. Furthermore, some of them, either individually or as part of molecular signatures, exhibit a significant association with clinical outcomes, such as disease-free (DFS) and overall survival (OS) [31,32]. For instance, increased levels of the lncRNA *HOTAIR*, previously reported in EOC tissues, showed an association with a surrogate DNA methylation profile that predicts poor survival in patients treated with carboplatin [33]. Recently published reviews provide additional examples of differentially expressed lncRNAs associated with OC prognosis [31,34,35]. In light of the growing body of evidence, it can be understood why lncRNAs could serve not only as prognostic and predictive markers but also as highly specific therapeutic targets. However, to date, few lncRNAs have been recognized as key players in the resistance to therapy in OC.

Overall, most of the well-characterized lncRNAs are associated with the gene expression regulation, commonly at transcriptional rather than at the post-transcriptional level. Importantly, lncRNAs may contribute to tumor biology by directly interacting with proteins of the epigenetic machinery or chromatin-remodeling complexes. The interplay between lncRNAs and these proteins results in epigenetic silencing or activation of target loci in a *cis* or *trans*-regulatory manner by altering chromatin structure, histone marks, or DNA methylation patterns of proximal regulatory elements. In this review, we revisit the opportunities to explore lncRNAs associated with the disruption of epigenetic reprogramming in OC. We also provided a guide for databases and tools that could help select disease-related lncRNAs for future functional studies and potential clinical applications.

2. lncRNA Genes and Their Expression Patterns

Although lncRNAs were previously recognized as noncoding genes, recent evidence has suggested that many of them contain small open reading frames that are strongly associated with ribosomes, which results in the translation of small peptides [36]. Several of these small peptides, often comprising fewer than 100 amino acids, are biologically functional and even implicated in cancer-suppressive or cancer-promoting activities [37]. Alternatively, some studies have linked mRNAs to coding-independent regulatory functions, blurring the differences between coding and noncoding transcripts, and highlighting the emergence of a new class of bifunctional RNAs [38].

lncRNAs are widely expressed in human tissues and, while less abundant, they are more tissue-specific than protein-coding genes [39,40]. Based on the genomic positions relative to protein-coding genes, lncRNAs can be classified into intergenic or intragenic, and sense, antisense, or bidirectional lncRNAs [41]. They share the same transcriptional machinery as protein-coding genes and usually undergo similar co-transcriptional and post-transcriptional processing events: 5'-capping, splicing, and 3'-polyadenylation [42]. Thus, standard RNA-seq protocols allow the identification of most lncRNA sequences [43]. In the evolutionary context, lncRNA exons evolve at a higher rate than protein-coding exons and lncRNA homologs exhibit limited sequence similarity in comparison with protein-coding genes [43]. As for genome diversity, lncRNAs surpass their protein-coding counterparts: lncRNA genes amount to nearly 60,000, against approximately 20,000 protein-coding genes [15,39].

From this myriad of lncRNAs, only a small part was deeply studied, and some patterns of action were proposed to depict their functions and help to predict the activities of other transcripts yet to be characterized. Molecularly, lncRNAs act by four mechanisms: as signals, reflecting biological contexts that are crucial for gene regulation in space and time; as scaffolds, binding to several proteins simultaneously and allowing the assembly of multi-subunit complexes; as guides, recruiting regulatory complexes to target loci in *cis* or *trans* via RNA-protein and RNA-DNA interactions; as decoys, regulating the availability of other biomolecules through sequestration [44]. A single

lncRNA frequently presents more than one mechanism of action. Most lncRNAs are exclusively localized in the nucleus, although some lncRNAs can occupy the chromatin and subnuclear domains or the cytoplasm. These localization patterns are possibly related to a particular lncRNA function [45]. Nuclear transcripts can perform their regulatory roles by interacting with epigenetic regulators and, consequently, affect DNA methylation, chromatin architecture, or histone marks deposition in *cis* and *trans* [46].

Besides the linear isoforms, lncRNA genes can generate transcripts with circular structures, called circRNAs [47]. The *CDKN2A/B* (cyclin-dependent kinase inhibitor 2A/B) locus, for instance, is known for its linear antisense ncRNA *CDKN2B-AS1* that interacts with the histone-modifying complexes Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2, respectively) and mediates transcriptional regulation. This mechanism was implicated in epigenetic silencing of tumor suppressor genes in the carcinogenesis process [48]. Apart from its linear isoform, the *CDKN2B-AS1* gene can produce circular transcripts by alternative splicing. The expression of these molecules was associated with atherosclerotic vascular disease risk [49]. Importantly, numerous circRNAs were found to be overexpressed in the serum of OC patients, whereas others, such as circ_0051240, circ-HIPK3 (homeodomain interacting protein kinase 3), and circRNA-UBAP2 (ubiquitin associated protein 2), were implicated in the control of OC cells malignant behavior [50,51]. CircRNAs are more stable in cells than their linear counterparts due to their covalent closed-loop structure. The lack of 5' end and poly(A)-tail come as a result of the circRNA biogenesis process, usually by lariat-driven circularization or back splicing, and avoid degradation by ribonucleases [52]. RNA-seq datasets obtained with poly(A)-independent methods are required for circRNA detection. However, the library preparation procedures still lack standardization, since there are several approaches to enrich the circRNA content (e.g., poly(A) depletion, rRNA depletion, and RNase R treatment), which lead to variability in results [53].

Pseudogenes are generally derived from their parental genes that underwent insertions, deletions, or nucleotide substitutions and lost their ability to encode for functional proteins. Therefore, pseudogenic transcripts can exert regulatory roles modulating the expression and/or the function of their cognate genes or unrelated coding genes [54]. Additionally, a subgroup of pseudogene loci can express lncRNAs, besides producing endogenous small interfering RNAs (siRNAs) [54]. The *PTENP1* (phosphatase and tensin homolog pseudogene 1) pseudogene, for example, encodes sense and antisense lncRNA transcripts that act to modulate the expression of its parental gene, the tumor suppressor *PTEN* (phosphatase and tensin homolog). Taking into account the experimental evidence of its involvement in tumor development and progression, either regulating *PTEN* or other genes, *PTENP1* can be considered a tumor suppressor [55].

The dysregulated expression of lncRNAs in cancer and their prominent spatial and temporal specificity compared with protein-coding genes reinforces their appeal as potential biomarkers and therapeutic targets [56]. Similar to lncRNAs, circRNAs present tissue/developmental stage-specificity and aberrant expression in cancer [57]. The increased stability, accompanied by their abundance in exosomes and body fluids, make circRNAs even more attractive for clinical applications [57,58]. As for pseudogenes, their expression is generally restricted to tumor tissues or increased in tumors compared to normal tissues [59]. In the fifth section, we present some invaluable resources to evaluate the abnormal expression levels and other features displayed by these noncoding transcripts in normal and pathological conditions.

3. lncRNAs and Their Association with Ovarian Cancer

In EOC, certain lncRNAs were found to be differentially expressed compared with benign and normal tissues. For instance, Wang et al. (2016) reported the up- or downregulation of 663 lncRNAs. The expression of the antisense lncRNA *RP11-597D13.9* was positively correlated with its nearby coding gene, *FAM198B* (family with sequence similarity 198 member B, also known as Golgi associated kinase 1B or *GASK1B*), which potentially participates in EOC progression [30]. Based on a systematic analysis of lncRNA and mRNA expression profiles from The Cancer Genome Atlas (TCGA), a platinum

resistance-specific lncRNA-mRNA network was identified in HGSOC [60]. A total of 35 lncRNAs and 270 mRNAs showed 124 significant lncRNA-mRNA co-expression relationships in this network. The study revealed that the lncRNAs involved in platinum resistance primarily regulate metabolic pathways and indicates the prognostic and therapeutic potential of lncRNAs in HGSOC [60]. Another study, including TCGA-HGSOC datasets, identified a 10-lncRNA prognostic signature that enabled to group the patients into low-, mid-, and high-risk groups, indicating significantly shorter OS and DFS for women in the last group [32].

lncRNA expression profiles of OC patients have been analyzed from GEO (Gene Expression Omnibus) datasets. These studies have used different bioinformatics approaches and included distinct datasets, with a little overlap between them. Song et al. (2018) reported a signature with seven lncRNAs (*RP11-126K1.6*, *ZBED3-AS1*, *RP11-439E19.10*, *RP11-348N5.7*, *RNF144A-AS1*, *GAS5*, and *F11-AS1*) [61], whereas Liu et al. (2017) identified a signature with eight lncRNAs (*ZFAS1*, *RP5-1061H20.5*, *RP11-489O18.1*, *RP11-16E12.1*, *LINC01514*, *TUG1*, *RP11-136I14.5*, and *CTD-2555A7.3*) [62], both able to predict the response of HGSOC patients to platinum-based chemotherapy [61,62]. A signature composed of seven different lncRNAs (*XR_948297*, *XR_947831*, *XR_938728*, *XR_938392*, *NR_103801*, *NR_073113*, and *NR_036503*) was significantly correlated with a poor response to treatment with paclitaxel in EOC patients [63]. Another signature comprising six lncRNAs (*RUNX1-IT1*, *MALAT1*, *H19*, *HOTAIRM1*, *LOC100190986*, and *AL132709.8*) was associated with recurrence in OC [64]. Overall, these studies revealed several lncRNAs linked to EOC via cancer-related functions and pathways, according to GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses.

To date, a few lncRNAs commonly associated with cancer were also involved in OC outcomes. A set of abnormally expressed lncRNAs, such as *H19* (*H19* imprinted maternally expressed transcript), *HOTAIR*, and *CDKN2B-AS1* was associated with patient survival [35]. The overexpression of the lncRNA *HOTAIR* in EOC tissues was positively correlated with the FIGO (International Federation of Gynecology and Obstetrics) stage, histological grade, lymph node metastasis, and inversely correlated with OS and DFS [65]. The knockdown of *HOTAIR* suppressed cell proliferation, reduced invasion, and restored cisplatin-sensitivity, specifically by enhancing chemotherapy-induced cytotoxicity and apoptosis in cisplatin-resistant OC cell lines [66]. Upregulated lncRNAs *CCAT2* (colon cancer-associated transcript 2), *LSINCT5* (long stress-induced noncoding transcript 5), *CERNA2* (competing endogenous lncRNA 2 for microRNA let-7b), *PVT1* (*Pvt1* oncogene), *UCA1* (urothelial cancer-associated 1), and *NEAT1* (nuclear paraspeckle assembly transcript 1) have also been implicated in cancer-promoting mechanisms in OC [67]. An additional example is *GAS5* (growth arrest-specific 5), which was found to be downregulated in OC and associated with poor prognosis [68]. Experimental assays inducing *GAS5* overexpression inhibited cell proliferation, migration, and invasion, besides promoting apoptosis [68].

The oncogenic and tumor suppressor actions of *HOTAIR* and *GAS5*, respectively, have been also described for other lncRNAs. *LEMD1-AS1* (*LEMD1* antisense RNA 1), for instance, inhibits OC progression by regulating an axis including miR-183-5p and TP53 (tumor protein p53) and leading to decreased cell proliferation, migration, and invasion [69]. *MAGI2-AS3* (*MAGI2* antisense RNA 3) downregulates MYC (MYC proto-oncogene, bHLH transcription factor) transcriptional activity via targeting the miR-525-5p/ *MXD1* (*MAX* dimerization protein 1) axis [70]. OC cell growth and motility are repressed as a consequence of *MAGI2-AS3* induced overexpression. Conversely, the silencing of *DLX6-AS1* (*distal-less homeobox 6 antisense RNA 1*) impaired proliferation, migration, and invasion abilities of OC cells, besides inducing their apoptosis and repressing tumor growth in vivo [25]. Increased levels of *DLX6-AS1* were detected in OC cells and tissues [25]. The authors demonstrated that *DLX6-AS1* acts by sponging the miR-195-5p and upregulating its miRNA-target FHL2 (four and a half LIM domains 2). Likewise, *TMPO-AS1* (thymopoietin antisense RNA 1) knockdown significantly suppressed OC cell proliferation, migration, invasion, and angiogenesis, also inhibiting tumorigenesis and angiogenesis in vivo [26]. *TMPO-AS1* was found to be upregulated in OC cell lines and clinical samples and exerts its oncogenic role by binding to E2F6 (*E2F* transcription factor 6) and modulating

its occupancy on the promoter region of *LCN2* (lipocalin 2), whose expression is activated. These examples highlight the potential of targeting lncRNAs for OC treatment.

In addition to the molecular signatures presented earlier, single lncRNAs have also been shown to affect response to therapy in OC, although the understanding of the underlying mechanisms is still in its infancy [31]. The well-known oncogenic lncRNAs *MALAT1*, *HOTAIR*, and *PANDAR* (promoter of *CDKN1A* antisense DNA damage-activated RNA) were associated with cisplatin resistance [71–73]. The silencing of *MALAT1* was demonstrated to enhance sensitivity to cisplatin due to the reduced downstream NOTCH1 (notch receptor 1) signaling, which led to the repression of the multidrug resistance gene *ABCC1* (ATP binding cassette subfamily C member 1) [72]. *HOTAIR* acts as a sponge for the miR-138-5p. The knockdown of *HOTAIR* was recently demonstrated to reverse cisplatin resistance due to the increased availability of the miR-138-5p repressing *EZH2* (enhancer of zeste 2 polycomb repressive complex 2 subunit) and *SIRT1* (sirtuin 1) [74]. Both *MALAT1* and *HOTAIR* have been suggested as potential therapeutic targets to restore platinum sensitivity. The overexpression of *PVT1* and *HOTAIR* were implicated in cisplatin resistance in cell line models by regulating apoptotic factors or activating the NF- κ B (nuclear factor kappa B) pathway, respectively [75,76]. Intriguingly, increased levels of *PVT1* after carboplatin-docetaxel treatment induced TP53 and TIMP1 (tissue inhibitor of metalloproteases-peptidase inhibitor 1) proteins expression concomitantly to decreased cell proliferation [77]. Acting by an unknown mechanism, the lncRNA *UCA1* promotes the expression of kinase *SRPK1* (SRSF protein kinase 1), which was associated with increased proliferation and reduced apoptosis of OC cells, favoring the development of cisplatin resistance [78]. Interestingly, Adriaens et al. (2016) suggested that targeting the lncRNA *NEAT1* can sensitize OC to platinum-based chemotherapy since its isoform 2 is involved in genomic integrity preservation by modulating ATR-Chk1 (ATM and Rad3-related-checkpoint 1) signaling and potentially affects double-stranded break (DSB) repair [79].

4. LncRNAs Involved in Epigenetic Mechanisms in Ovarian Cancer

The role of epigenetic machinery elements generally fits into one of three patterns of activity: (i) the writers can transfer chemical groups to nucleotide bases in DNA or amino acid residues in histone tails, (ii) the erasers are capable of removing these marks, and (iii) the readers are specialized in recognizing the DNA and histone epigenetic modifications. The writers include DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), and histone acetyltransferases (HATs), while DNA demethylases, histone demethylases (HDMs), and histone deacetylases (HDACs) are their eraser counterparts. These molecules usually act in gene expression regulation by associating with lncRNAs and transcription factors [80,81]. In this section, we provide examples of lncRNAs whose interaction with proteins of the epigenetic machinery is supported by strong experimental evidence and potentially influence OC outcomes (Figure 1). These examples were obtained by searching either the lncRNA databases listed or from the published literature, i.e., referring to recent studies not included in databases (Table 1).

The knowledge of the interplay between lncRNAs, epigenetic machinery, and transcriptional regulation could significantly expand our understanding of how lncRNA dysregulation contributes to the epigenome and transcriptome alterations and, consequently, to tumor initiation and progression. LncRNAs participate in the epigenetic regulation by several mechanisms, including (i) interaction with DNMTs, guiding these proteins to a specific locus, leading to the promoter methylation and repression of tumor suppressor genes; (ii) by changing the nucleosome positioning and modifying chromatin accessibility to the transcriptional machinery; (iii) by recruiting histone-modifying enzymes in *cis* or *trans*. Another possible mechanism considers that lncRNAs can recruit demethylases and/or acetylases to the promoter region of oncogenes, and consequently activate them [82,83].

Hu et al. (2014) demonstrated the interaction between the lncRNA *FALEC* (focally amplified long noncoding RNA in epithelial cancer), also known as *FAL1*, and BMI1 (BMI1 proto-oncogene), a core component of PRC1. The authors reported the association of *FAL1* expression levels to the stabilization of BMI1 protein in human OC cell lines [84].

Table 1. Representative lncRNAs associated with disruption of epigenetic mechanisms in ovarian cancer.

LncRNA	Regulatory Mechanism	Target	Source	Refs.
<i>FAL1 (FALEC)</i>	Stabilization of BMI1 protein and PRC1-mediated deposition of repressive histone marks	<i>CDKN1A</i>	LncTarD and EVLncRNAs databases	[84,85]
<i>PVT1</i>	EZH2 recruitment	miR-214	LncTarD database	[86]
<i>TARID</i>	Recruitment of GADD45A and TET-dependent DNA demethylation	<i>TCF21</i>	LncTarD database	[87,88]
<i>LINC01210</i>	EZH2 recruitment and PRC2-mediated deposition of repressive histone marks	<i>KLF4</i>	Literature mining	[89]
<i>TP73-AS1</i>	EZH2 recruitment and PRC2-mediated deposition of repressive histone marks	<i>CDKN1A</i>	Literature mining	[90]
<i>UNC5B-AS1</i>	EZH2 recruitment and PRC2-mediated deposition of repressive histone marks	<i>NDRG2</i>	Literature mining	[91]
<i>ABHD11-AS1</i>	EZH2 recruitment and PRC2-mediated deposition of repressive histone marks	<i>TIMP2</i>	Literature mining	[92]

ABHD11-AS1: ABHD11 antisense RNA 1 (tail to tail); BMI1: BMI1 proto-oncogene, polycomb ring finger; *CDKN1A*: cyclin-dependent kinase inhibitor 1A; EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit; *FALEC*: focally amplified long noncoding RNA in epithelial cancer; GADD45A: growth arrest and DNA damage-inducible alpha; *KLF4*: Kruppel like factor 4; *LINC01210*: long intergenic nonprotein coding RNA 1210; *NDRG2*: NDRG family member 2; PRC1: Polycomb Repressive Complex 1; PRC2: Polycomb Repressive Complex 2; *PVT1*: Pvt1 oncogene; *TARID*: TCF21 antisense RNA inducing promoter demethylation; *TCF21*: transcription factor 21; TET: ten-eleven translocation family; *TIMP2*: TIMP metalloproteinase inhibitor 2; *TP73-AS1*: TP73 antisense RNA 1; *UNC5B-AS1*: UNC5B antisense RNA 1.

The PRC1 histone-modifying complex is known for binding the promoter regions of its target genes and mediating the deposition of one ubiquitin group to histone H2A at lysine 119 (H2AK119ub) [93]. However, the significance of this mark to the transcriptional repression of target genes is not yet fully understood. A novel mechanism was recently described for BMI1, which works in a PRC1-independent and non-epigenetic manner to regulate androgen receptor protein in castration-resistant prostate cancer [94]. Nevertheless, in Hu et al. (2014) study, the depletion of *FAL1* was correlated with the upregulation of *CDKN1A* (cyclin-dependent kinase inhibitor 1A). Experimental evidence suggests that the underlying mechanism involves, at least in part, the modulation of BMI1 occupancy and

ubiquitination of H2AK119 on the promoter region of *CDKN1A*. The p21 protein encoded by *CDKN1A* promotes cell-cycle arrest and senescence. In vitro and in vivo studies demonstrated that both these processes were positively regulated by *FAL1* knockdown. Thus, *FAL1* has an oncogenic function in OC partly exerted by the epigenetic regulation of p21 expression levels, hinting at potential implications for cancer therapy [84].

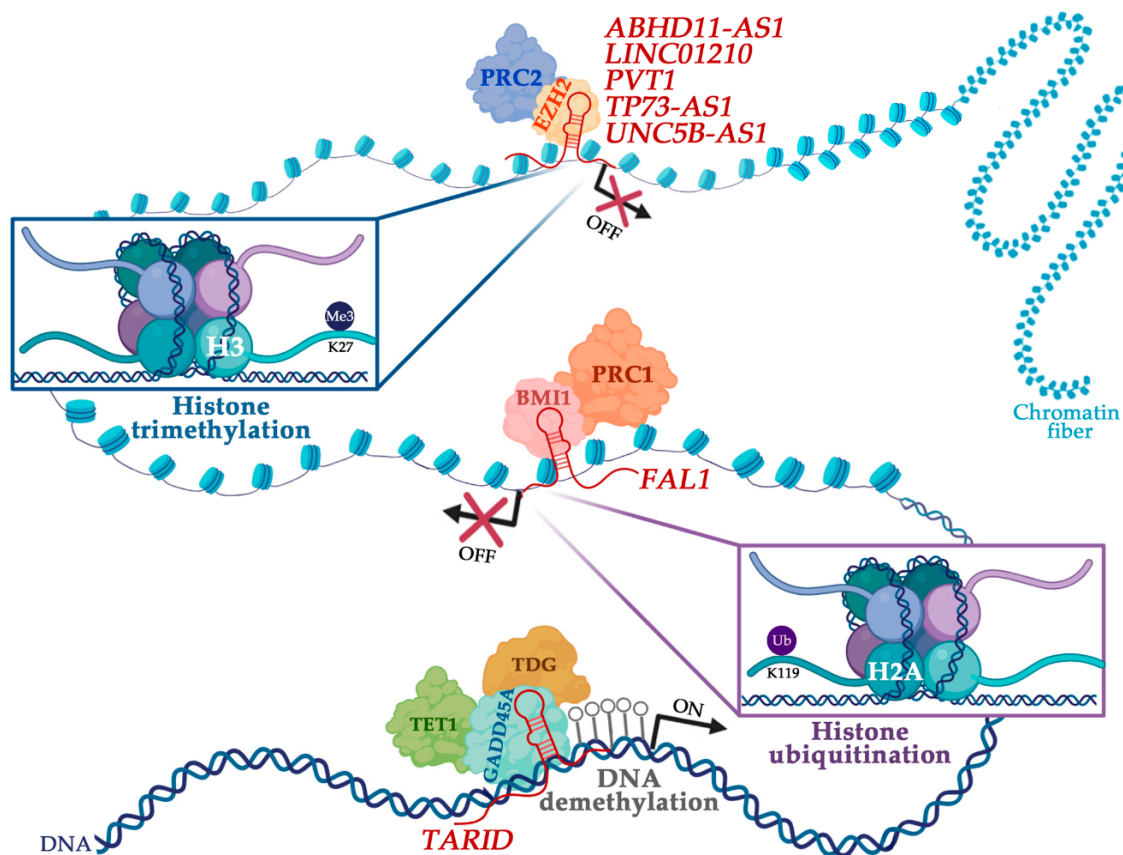


Figure 1. Long noncoding RNAs (lncRNAs) as epigenetic regulators in ovarian cancer. The representative lncRNAs implicated in each regulatory mechanism are shown in dark red. In the top and center, the interplay between lncRNAs and repressive histone-modifying complexes (Polycomb Repressive Complexes 2 and 1, respectively) are illustrated. The functional relationship between the lncRNA *TARID* (TCF21 antisense RNA inducing promoter demethylation) and the DNA demethylation is presented at the bottom of the figure. (Created with biorender.com).

The lncRNA *PVT1* recruits the protein *EZH2* to the promoter of *miR-214* [86]. *EZH2* is one of two alternative catalytic subunits of *PRC2* and is required, along with two other core components (*SUZ12* polycomb repressive complex 2 subunit and embryonic ectoderm development), to produce the repressive histone modifications that characterize *PRC2* activity: mono-, di-, and trimethylation of histone H3 at lysine 27. Under physiological conditions, the complex is recruited to unmethylated CpG islands and ensures the maintenance of previously established transcriptional silencing states, contributing to preserving cell identity [95]. In EOC, the upregulation of *EZH2* has been reported by several studies and promotes proliferation, invasion, metastasis, and angiogenesis, besides its association with cisplatin resistance in OC cells [96].

In OC, both overexpression and downregulation of *miR-214* have been described [97]. In the first case, this miRNA was implicated in reducing the cisplatin-induced apoptosis by targeting *PTEN*, whereas downregulation was linked to cancer cell stem-like properties and conversion of normal fibroblasts into cancer-associated fibroblasts [97]. Chen and colleagues identified increased *PVT1*

and decreased miR-214 expression levels in EOC tissue samples and OC cell lines in comparison to normal tissue samples and human primary ovarian cells, respectively. In vitro results demonstrated the interaction between EZH2 and *PVT1* and the association of the lncRNA with proliferation, migration, and invasion [86].

Additional examples of lncRNAs capable of recruiting EZH2 to transcriptionally repress their target genes are *LINC01210* (long intergenic nonprotein coding RNA 1210), *TP73-AS1* (TP73 antisense RNA 1), *UNC5B-AS1* (UNC5B antisense RNA 1), and *ABHD11-AS1* (ABHD11 antisense RNA 1 (tail to tail)). Increased expression levels of *LINC01210* were reported in OC tissues, notably in metastatic or advanced stages of the disease, which were correlated with poor prognosis [89]. This lncRNA epigenetically downregulates the transcription factor KLF4 (Krüppel-like factor 4), favoring cell proliferation, invasion, and migration [89]. In OC, induced KLF4 overexpression promoted apoptosis and improved the efficacy of chemotherapy drugs (cisplatin and paclitaxel) [98]. Similar to the lncRNA *FAL1*, *TP73-AS1* mediates epigenetic repression of the tumor suppressor *CDKN1A*, although through with EZH2 instead of BMI1. *TP73-AS1* is upregulated in EOC tissues and cell lines, correlating with poor prognosis, and facilitating tumor progression by targeting *CDKN1A* [90].

The lncRNA *UNC5B-AS1* and its target gene *NDRG2* (NDRG family member 2) have been poorly explored in OC. *NDRG2* has been associated with tumor suppression in several cancer types and was found to be downregulated in OC tissues and cell lines [91]. In this context, *UNC5B-AS1* recruits EZH2 to inhibit *NDRG2* transcription, promotes cell proliferation, and impairs apoptosis [91]. To confirm bioinformatic predictions, *ABHD11-AS1* was experimentally proven to interact with EZH2 leading to the epigenetic repression of *TIMP2* (TIMP metalloproteinase inhibitor 2) and further promoting invasion and metastasis [92]. This evidence sheds light on the function of *ABHD11-AS1* in OC, albeit the inverse correlation between *EZH2* and *TIMP2* expression levels had already been detected in EOC tissues [99]. *TIMP2* is a member of the TIMP gene family, which encodes proteins that inhibit the activity of matrix metalloproteinases. The metalloproteinases degrade the extracellular matrix and facilitate metastasis initiation. Unsurprisingly, *TIMP2* aberrant expression promotes cell growth and invasion in several malignancies [92].

The antisense lncRNA *TARID* (TCF21 antisense RNA inducing promoter demethylation) mediates demethylation of the protein-coding gene *TCF21* (transcription factor 21), a tumor suppressor that is transcribed in the sense orientation. In OC samples, the CpG islands associated with the *TCF21* and *TARID* promoter regions are hypermethylated, and both genes are repressed. In contrast, in normal ovarian epithelium, the promoters are unmethylated, and both transcripts are expressed. It was shown that *TARID* binds to the promoter of its target gene and recruits GADD45A (growth arrest and DNA damage-inducible alpha), a stress response protein [88]. *GADD45A* expression level is commonly decreased in different cancer types. Interestingly, one functional single nucleotide polymorphism mapped to this gene is related to significantly decreased *GADD45A* mRNA levels and was implicated in OC susceptibility and prognosis [100]. Recent evidence has suggested that GADD45A recognizes the DNA-lncRNA hybrid formed at the CpG island of the *TCF21* promoter, rather than its sequence, and attracts the DNA demethylation machinery to activate the transcription of this gene. Consequently, GADD45A was proposed to be an epigenetic reader [101]. It recruits TDG (thymine DNA glycosylase), a component of the deamination-base excision repair machinery (BER), and proteins of the TET (ten-eleven translocation) family to the *TCF21* promoter [88]. TETs are epigenetic erasers known for their role as DNA demethylases in mammals; they oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and afterward to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC). The residues of 5fC and 5caC can be replaced by unmodified cytosine following excision by TDG and repair of the abasic site by BER. This process is called active modification-active removal and occurs independently of DNA replication [102].

The examples of epigenetic mechanisms mediated by lncRNAs presented here could help to uncover new regulatory interactions between lncRNAs and epigenetic machinery components. Considering the PRC2 promiscuous binding to RNAs along with the estimate that more than 20% of

human lncRNAs are associated with PRCs and chromatin-remodeling complexes, the recruitment of EZH2 and other less-studied mechanisms described herein are likely to apply to some of the still uncharacterized lncRNAs as well [103,104]. Furthermore, our portrayal of lncRNAs associated with epigenetic machinery elements provide frameworks that could be explored in future studies to characterize the function of OC-associated lncRNAs.

5. A Concise Guide to Exploring lncRNA Databases

The significant amount of data generated using low- and high-throughput methods for the identification and functional characterization of lncRNAs has been organized and updated in many user-friendly databases. Overall, these comprehensive databases provide basic information such as lncRNA annotation, lncRNA–target interactions, the affected biological processes, and lncRNA-mediated regulatory mechanisms in human diseases (transcriptional regulation, epigenetic regulation, chromatin looping, competing endogenous RNA or miRNA sponge, relationships with mRNAs and proteins). They are useful for analyzing and defining the best candidates for further investigations when considering the involvement of lncRNAs in human diseases. The databases summarized herein are publicly available and have information collected on the association between lncRNAs and human diseases. Thus, they are invaluable resources to discover novel biomarkers and potential therapeutic targets for ovarian tumors.

Concurrently, the selected databases are focused on different aspects of lncRNA biology. Therefore, we grouped them into three major categories that can be used as a guide to browse these resources. Based on their main focuses, i.e., annotation of lncRNAs, regulatory functions, or association with human diseases and phenotypes, we summarized this set of representative databases in Table 2. Table S1 includes features of these databases, such as data sources, coverage, and other relevant information. For instance, NONCODE, LNCipedia, LncRNAWiki, EVLncRNAs, LncRBase, and LncBook are mainly dedicated to annotating lncRNAs [105–110]. Although the data sources of these databases partly overlap, some of them also integrate information on lncRNAs from different species, their functions, sequence conservation, and other details. Most importantly, all of them include associations between lncRNAs and human cancers. Among these resources, LncBook is the most comprehensive in terms of the number of human lncRNAs, which amounts to 268,848 transcripts and 140,356 genes. Considering the disease-related lncRNAs gathered by this knowledgebase, *HOTAIR*, *MALAT1*, *H19*, *MEG3* (maternally expressed 3), *CDKN2B-AS1*, *PVT1*, *NEAT1*, and *GAS5* are some of the most studied. Each of these lncRNAs has been associated with at least 30 diseases, including OC [108].

Table 2. Summary of lncRNA databases (last accessed on July, 2020).

Database	Aim	Website	Refs.
lncRNA annotation			
NONCODE v5.0	Collects and annotates a comprehensive set of ncRNAs, especially lncRNAs.	http://www.noncode.org/	[105]
LNCipedia 5.2	Collects sequences and annotation data for a comprehensive set of lncRNA transcripts.	https://lncipedia.org	[107,111]
LncRNAWiki	Collects and integrates comprehensive information on human lncRNAs relying on collaborative curation.	http://lncrna.big.ac.cn	[110,112]

Table 2. Cont.

Database	Aim	Website	Refs.
EVLncRNAs	Collects lncRNAs that were validated by low-throughput experiments.	http://biophy.dzu.edu.cn/EVLncRNAs	[109]
LncRBase	Provides information on a comprehensive set of lncRNAs, ranging from basic transcript features to additional details on their genomic context.	http://bicresources.jcbose.ac.in/zhumur/lncbase	[106]
LncBook	Collects a comprehensive set of human lncRNAs and provides multi-omics data integration, functional annotation, and disease association for these transcripts.	http://bigd.big.ac.cn/lncbook	[108,113]
lncRNA regulatory functions (interactions, mechanisms, and effects)			
ENCORI	Integrates multi-dimensional sequencing data to provide a comprehensive collection of RNA–RNA and protein–RNA interaction networks.	http://starbase.sysu.edu.cn/	[114,115]
LncRNA2Target v2.0	Collects lncRNA–target associations supported by high- and low-throughput methods.	http://123.59.132.21/lncrna2target/	[116]
LncTarD	Collects and illustrates experimentally supported lncRNA–target regulations in human diseases.	http://bio-bigdata.hrbmu.edu.cn/LncTarD	[117]
NPInter v4.0	Integrates literature reported evidence and high-throughput data processing to collect and illustrate functional interactions between ncRNAs and biomolecules.	http://bigdata.ibp.ac.cn/npinter	[118]
LncACTdb 2.0	Collects and illustrates information on ceRNA relationships between different types of RNAs in diverse diseases.	http://www.bio-bigdata.net/LncACTdb/	[119]
miRSponge	Collects experimentally validated miRNA sponges and ceRNA interactions.	http://bio-bigdata.hrbmu.edu.cn/miRSponge/	[120]

Table 2. Cont.

Database	Aim	Website	Refs.
Co-LncRNA	Allows the identification of lncRNA coexpressed protein-coding genes and the assessment of the effects of individual or multiple lncRNAs in GO annotations and KEGG pathways.	http://bio-bigdata.hrbmu.edu.cn/Co-LncRNA/	[121]
Lnc2Meth	Integrates experimental and computational evidence to provide information on regulatory relationships between lncRNAs and DNA methylation in diverse human diseases.	http://bio-bigdata.hrbmu.edu.cn/Lnc2Meth/	[122]
lncRNA–disease associations			
LncRNADisease 2.0	Integrates experimental and computational evidence to provide information on lncRNA/circRNA–disease associations, including causal relationships.	http://www.rnanut.net/lncrnadisease/	[123,124]
Lnc2Cancer v3.0	Collects and integrates experimentally supported associations between lncRNAs and cancer subtypes.	http://www.bio-bigdata.net/lnc2cancer/	[125,126]
MNDR v2.0	Collects and integrates experimentally supported and predicted ncRNA–disease associations in mammals.	http://www.rna-society.org/mndr/	[127]
ncRPheno	Collects comprehensive ncRNA–disease association data and offers web applications to facilitate analysis and visualization of this data.	http://lilab2.sysu.edu.cn/ncrpheno	[128]
Lnc2Atlas	Integrates multiple methods and data sources to collect quantitative associations between lncRNAs and cancer.	http://lnc2atlas.bioinfotech.org/	[129]
CSCD	Collects and integrates information on cancer-specific circRNAs.	http://gb.whu.edu.cn/CSCD	[130]
Circ2Disease	Collects information on experimentally supported circRNAs in human diseases.	http://bioinformatics.zju.edu.cn/Circ2Disease/index.html	[131]

Table 2. Cont.

Database	Aim	Website	Refs.
CircR2Disease	Collects information on experimentally validated associations between circRNAs and diseases.	http://bioinfo.snnu.edu.cn/CircR2Disease/	[132,133]
MiOncoCirc	Collects data on circRNA formation and abundance in cancer.	https://mioncocirc.github.io/	[134]

The regulatory functions of lncRNAs could be especially explored in databases such as ENCORI, LncRNA2Target, LncTarD, and NPInter, which dive into interactions between these transcripts and different types of biomolecules [114,116–118]. Two other databases, LncACTdb and miRSponge, curate miRNA–sponge relationships [119,120]. Endogenous transcripts that share the same miRNA-binding sites can compete for the corresponding miRNA and regulate each other post-transcriptionally by titrating miRNA availability [135]. These transcripts, called competing endogenous RNA (ceRNAs), are comprised of noncoding and protein-coding RNAs, and the role of these regulators in cancer can be found in Salmena et al. (2011) and Yang et al. (2016) [136,137]. ENCORI provides a comprehensive collection of RNA–RNA and protein–RNA interaction networks. Lnc2Target collects lncRNA–target associations validated by high- and low-throughput methods. Emphasizing the contribution of lncRNAs to pathogenesis in humans, LncTarD only collects disease-related lncRNA–target regulations. On the other hand, NPInter curates functional interactions between ncRNAs and DNA, proteins, or other types of RNA across numerous organisms. Lately, the concern about offering tools that facilitate the analyses of the information available in databases has been raised, and each of these resources, except for LncRNA2Target and miRSponge, contains applications that illustrate the interactions between lncRNAs and their putative partners. Co-LncRNA and Lnc2Meth are also devoted to the elucidation of regulatory roles of human lncRNAs, the first analyzing downstream effects, and the second focusing on a mechanism [121,122]. Co-LncRNA employs RNA-seq data to assess the effects of individual or multiple lncRNAs in GO annotations and KEGG pathways, whereas Lnc2Meth links lncRNAs to DNA methylation status in several diseases.

Other databases were specially built to provide information on lncRNA–disease associations, such as LncRNADisease, Lnc2Cancer, MNDR, ncRPheno, and Lnc2Atlas [123,125,127–129]. LncRNADisease combines experimental and bioinformatic evidence to provide information on relationships between lncRNAs or circRNAs and diseases. It is the first lncRNA–disease association database that documents causal relationships, mostly cancer-related, which were identified based on strong experimental evidence from in vitro and/or in vivo models [124]. Lnc2Cancer strictly gathers associations supported by experimental evidence and human cancer subtypes, whereas MNDR is dedicated to both experimentally validated and predicted ncRNA–disease associations across six mammalian species. ncRPheno provides several web applications to facilitate the analysis and visualization of ncRNA–disease associations. Lnc2Atlas relies on three scoring methods to collect associations between lncRNAs and cancer. Additionally, some of the resources exemplified by CSCD, Circ2Disease, CircR2Disease, and MiOncoCirc cover the involvement of circRNAs in diseases exclusively [130–132,134]. MiOncoCirc is the first circRNA database to include datasets derived from clinical tumor samples. All MiOncoCirc samples were processed with the poly(A)-independent exome capture RNA-seq protocol, an approach that was proven to be more reliable than the often-used RNase R and rRNA removal methods. This database supports the identification of tissue-specific transcripts that could be further explored as biomarkers in cancer [134].

Bioinformatic tools also help to unveil the association between lncRNAs and different cancer types. The Atlas of Noncoding RNAs in Cancer (TANRIC) data portal is a platform for interactive analysis of clinical and genomic data coupled to lncRNA expression in cancer, including RNA-seq data

from TCGA, independent studies, and cell lines from Cancer Cell Line Encyclopedia [138]. OncoLnc is an online resource that integrates survival data from TCGA and RNA-seq expression data for mRNAs, miRNAs, and lncRNAs to enable the exploration of survival correlations, besides allowing the download of the data used in the analyses [139]. Finally, LncDisease is a standalone software that relies on a sequence-based method to predict associations between lncRNAs and human diseases. The analysis involves two steps: (i) the prediction of miRNAs that interact with the inputted lncRNAs and (ii) the disease enrichment analysis of the predicted miRNAs, providing a list of significant diseases potentially associated with lncRNAs [140].

6. Conclusions and Perspectives

The altered expression profiles of lncRNAs in cancer, their tissue specificity, and regulatory roles highlight the importance of elucidating lncRNAs functions for new advances in OC diagnosis, prognosis, and therapy. Despite the promising findings, greatly accelerated by high-throughput and *in silico* approaches, and the large number of lncRNA in the human genome, few cancer-related lncRNAs have been characterized, annotated, and explored to shed light on their potential for clinical applications.

Currently, the development of epi-drugs, comprising compounds targeting enzymes involved in epigenetic regulations, has been limited by the lack of biomarkers for proper patient stratification for clinical trials [141]. Furthermore, interfering with a single epigenetic alteration can lead to global and local effects on chromatin conformation affecting several biological processes and pathways [142]. The regulation of the epigenetic machinery by multiple lncRNAs includes an additional layer of complexity. These transcripts can serve a dual purpose: allowing specific interference with selected targets and a more robust evaluation of patients' responses to the treatment with epi-drugs. The technology for silencing specific lncRNA already exists; however, our knowledge of the widespread functions and interactions of lncRNAs is still limited.

In order to target lncRNAs for further investigations and the development of novel therapeutic strategies, ASOs (antisense nucleotides), siRNAs, and the CRISPR (clustered regularly interspaced short palindromic repeats) system are some of the available methodologies [21]. Each of these approaches offer different routes to achieve the lncRNA modulation and particular advantages and pitfalls that should be appreciated according to the features of the intended targets. ASOs, for instance, are single-stranded deoxyribonucleotide molecules that can hybridize with complementary lncRNAs and attract RNase H to degrade the target RNA. These oligonucleotides can be modified to improve their intracellular stability and are equally successful in silencing nuclear and cytoplasmic transcripts [143]. In contrast, siRNAs are less effective for the depletion of nuclear lncRNAs and, consequently, are preferable for cytoplasmic applications [144]. The CRISPR system can be used to knockout the target lncRNA genes when coupled with endonuclease Cas9, which produces DSBs in specific DNA sequences, or to repress the target lncRNA transcription when coupled with catalytically dead Cas9 (dCas9) fused to KRAB (Kruppel-associated box) chromatin modifier domain [143,145]. Hopefully, the information and guidelines provided here can help with the design of future studies employing the aforementioned methods to gain more insight into how lncRNAs can be exploited as biomarkers and therapeutic targets in OC.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/9/2701/s1>: Table S1: Publicly available lncRNA databases and their main features (last accessed on July 2020).

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5. Capítulo 2

O capítulo 2 destaca o artigo publicado no periódico *Cancers* (ISSN 2072-6694):

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O artigo contemplado no capítulo 2 incluiu análises transcriptômicas e epigenômicas que revelaram um conjunto de lncRNAs diferencialmente expressos e associados a dinucleotídeos CpG diferencialmente metilados em linhagens celulares de OC intrinsecamente resistentes à carboplatina e subpopulações celulares cuja resistência foi induzida. Esses achados foram comparados com dados públicos de RNA-Seq de pacientes com HGSOC e o lncRNA *SNHG12* se destacou por exibir correlação inversa entre os níveis de expressão e metilação do DNA nas linhagens celulares e expressão aumentada tanto nas linhagens celulares quanto em amostras de câncer de ovário de pacientes resistentes à carboplatina. Após o silenciamento de *SNHG12*, foi observado um aumento na sensibilidade à carboplatina nas linhagens celulares. Vários lncRNAs da família *SNHG* (*small nucleolar RNA host genes*) são reconhecidos por suas funções oncogênicas, exercidas por meio de diversas interações com moléculas de DNA, RNA e proteínas^{81,82}. Nosso estudo detectou que os genes que flanqueiam o *locus* do *SNHG12* (*PHACTR4*, *RCC1*, *TAF1* e *GMEB1*) apresentavam expressão diminuída nas linhagens celulares de OC resistentes à carboplatina. Esses resultados, combinados com previsões anteriores da literatura, sugeriram que *SNHG12* poderia modular a expressão dos genes vizinhos, atuando como guia para fatores de transcrição e outras proteínas responsáveis por

regular a expressão gênica. Dados da literatura mostraram que *SNHG12* contribui para a evasão da resposta imune pelo tumor ao recrutar NF- κ B e promover a expressão de *IL6R* (*interleukin 6 receptor*) no OC ⁸³. Sun e Fan (2019) demonstraram que *SNHG12* favorece a proliferação e a migração em linhagens celulares derivadas de OC por meio da ligação com o miRNA-129, impedindo que este regule negativamente os níveis do fator de transcrição SOX4 (*SRY-box transcription factor 4*) ⁸⁴. Contudo, ainda não foi elucidado o mecanismo pelo qual *SNHG12* promove a resistência à quimioterapia no OC. Um estudo com CLIP-seq (do inglês: *Crosslinking Immunoprecipitation and High-throughput Sequencing*), recuperado pela ferramenta NPInter, predisse a interação entre *SNHG12* e a desacetilase de histonas HDAC9 (*histone deacetylase 9*) ⁸⁵. Essas evidências sugerem a interação de *SNHG12* com proteínas da maquinaria epigenética, o que poderia ser um dos mecanismos por meio dos quais esse lncRNA atua na resposta ao tratamento.

Article

The Long Non-Coding RNA *SNHG12* as a Mediator of Carboplatin Resistance in Ovarian Cancer via Epigenetic Mechanisms

Cecilie Abildgaard ^{1,2}, Luisa Matos do Canto ¹ , Cláudia Aparecida Rainho ³ , Fabio Albuquerque Marchi ⁴ , Naiade Calanca ^{1,3} , Marianne Waldstrøm ^{5,6}, Karina Dahl Steffensen ^{2,6}  and Silvia Regina Rogatto ^{1,6,*} 

¹ Department of Clinical Genetics, Lillebaelt University Hospital of Southern Denmark, 7100 Vejle, Denmark; cecilieabild@gmail.com (C.A.); luisa.matos.do.canto.alvim@rsyd.dk (L.M.d.C.); naiade.calanca@unesp.br (N.C.)

² Department of Oncology, Lillebaelt University Hospital of Southern Denmark, 7100 Vejle, Denmark; karina.dahl.steffensen@rsyd.dk

³ Department of Chemical and Biological Sciences, Institute of Biosciences, São Paulo State University (UNESP), Botucatu 18618-689, Brazil; claudia.rainho@unesp.br

⁴ International Research Center, CIPE—C. Camargo Cancer Center, São Paulo 04002-010, Brazil; biomarchi@gmail.com

⁵ Department of Pathology, Lillebaelt University Hospital of Southern Denmark, 7100 Vejle, Denmark; marianne.waldstroem@rsyd.dk

⁶ Institute of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark, 5000 Odense, Denmark

* Correspondence: silvia.regina.rogatto@rsyd.dk; Tel.: +45-7940-6669



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Simple Summary: Epithelial ovarian cancer is a lethal malignancy in which recurrence and therapy resistance are the major causes of death. We investigated the transcriptome and DNA methylation profile of ovarian cancer cell lines sensitive and resistant to carboplatin, aiming to identify genes associated with therapy resistance. We focused on long non-coding RNAs (lncRNAs), known as epigenetic regulators of several cellular and biological processes. We found 11 lncRNAs associated with carboplatin resistance, including *SNHG12* (small nucleolar RNA host gene 12), also confirmed in an external dataset (The Cancer Genome Atlas). *SNHG12* gene silencing increased the sensitivity to carboplatin, giving evidence that this lncRNA contributes to resistance to carboplatin in ovarian cancer cell lines. We also demonstrated that *SNHG12* could control the expression of nearby genes probably by altering epigenetic markers and modifying the transcript levels.

Abstract: Genetic and epigenetic changes contribute to intratumor heterogeneity and chemotherapy resistance in several tumor types. lncRNAs have been implicated, directly or indirectly, in the epigenetic regulation of gene expression. We investigated lncRNAs that potentially mediate carboplatin-resistance of cell subpopulations, influencing the progression of ovarian cancer (OC). Four carboplatin-sensitive OC cell lines (IGROV1, OVCAR3, OVCAR4, and OVCAR5), their derivative resistant cells, and two inherently carboplatin-resistant cell lines (OVCAR8 and Ovc316) were subjected to RNA sequencing and global DNA methylation analysis. Integrative and cross-validation analyses were performed using external (The Cancer Genome Atlas, TCGA dataset, $n = 111$ OC samples) and internal datasets ($n = 39$ OC samples) to identify lncRNA candidates. A total of 4255 differentially expressed genes (DEGs) and 14529 differentially methylated CpG positions (DMPs) were identified comparing sensitive and resistant OC cell lines. The comparison of DEGs between OC cell lines and TCGA-OC dataset revealed 570 genes, including 50 lncRNAs, associated with carboplatin resistance. Eleven lncRNAs showed DMPs, including the *SNHG12*. Knockdown of *SNHG12* in Ovc316 and OVCAR8 cells increased their sensitivity to carboplatin. The results suggest that the lncRNA *SNHG12* contributes to carboplatin resistance in OC and is a potential therapeutic target. We demonstrated that *SNHG12* is functionally related to epigenetic mechanisms.

Keywords: ovarian cancer; lncRNA; drug resistance; chemotherapy; DNA methylation; transcriptomic analysis

1. Introduction

Ovarian cancer (OC) is one of the most lethal malignancies in women and accounts for more than 200,000 deaths worldwide annually [1]. OC is histologically divided into at least five subtypes showing distinct etiological, morphological, clinical, genetic, and epigenetic aspects [2,3]. Epithelial ovarian cancer (EOC) accounts for ~90% of cases and includes serous, endometrioid, clear cell, and mucinous carcinomas. High-grade serous OC is the most commonly diagnosed, and in more than 70% of cases presents advanced stages (International Federation of Gynecology and Obstetrics, FIGO, stage III or IV) [2,4,5].

Primary care of OC patients is based on surgery and adjuvant chemotherapy. Despite the variety of OC subtypes, pointing in the direction that each subtype should potentially be treated differently, OC is treated as a single disease. Standard first-line treatment includes primary cytoreductive surgery followed by adjuvant chemotherapy with platinum-based drugs, namely carboplatin or cisplatin, and a taxane, such as paclitaxel or docetaxel [6,7]. Initially, more than 80% of the women benefited from the first-line treatment. However, within two years from diagnosis, most patients with advanced disease experience recurrence due to treatment resistance [7]. Ovarian cancer that recurs within six months of completion of initial therapy is considered “platinum-resistant”, while OC that recurs after six months of completion of initial therapy is considered “platinum-sensitive” [8]. In advanced stages of the disease, the efficacy of different therapies decreases with each recurrence, and the patients face an abysmal prognosis of less than 25% 5-year survival [9–11]. Cancer recurrence and therapy resistance are considered the major causes of death. Therefore, it is critically urgent to understand the pathobiology of this aggressive disease better and provide more precise and effective treatments.

Among the drugs used in recurrent OC, PARP (poly- (adenosine diphosphate-ribose) polymerase) inhibitors have demonstrated prognostic impact in patients with pathogenic/likely pathogenic *BRCA1/BRCA2* variants or genomic instability [12,13]. After primary chemotherapy, PARP inhibitors have also been used as maintenance therapy [12]. The anti-angiogenic monoclonal antibody bevacizumab, which binds to vascular endothelial growth factor, has been approved to treat EOC, but no significant difference in overall survival and limited improvement in progression-free survival has been described [14]. Immunological therapies, anti-angiogenic agents, combination therapies, and treatment based on genetic alterations (targeted therapies) have been intensively studied to improve the survival and quality of life of OC patients.

The heterogeneity of the disease and the molecular complexity of the genetic and epigenetic alterations promoted during tumor progression or due to the selective pressure caused by the treatment is rarely captured and taken into consideration in the clinical setting [15]. The molecular alterations leading to platinum resistance rarely involve single nucleotide mutations in known drivers or resistance-associated genes. Instead, the resistance appears to arise from a number of highly patient-specific alterations such as aberrant DNA methylation, gene amplifications, reversion of *BRCA1/2* (*BRCA1* and *BRCA2* DNA repair associated) mutations, chromosomal rearrangements, and differential expression of non-coding RNAs (ncRNAs) [10,16–18].

The ncRNAs, which are involved in transcriptional and post-transcriptional regulation, constitute an additional level of complexity in cancer drug resistance. Among them, long non-coding RNAs (lncRNA) have been drawing attention for their function as regulators of transcription and chromatin remodeling. LncRNAs include transcripts longer than 200 nucleotides that form complex structures that interact with DNA, RNA, or proteins to modulate gene expression and cellular signaling [19]. LncRNAs are expressed in lower levels compared to mRNA, control the expression pattern during the development and cell differentiation, and are often cell-type and cancer-type specific [20]. For instance, *SNHG10* (small nucleolar RNA host gene 10) overexpression significantly suppressed the tumorigenesis and epithelial-mesenchymal transition (EMT) of EOC [21]. *SNHG10*/miR-200a-3p/*BIN1* axis was reported as a predictive biomarker and therapeutic target in OC [21]. The lncRNA *HOTAIR* has been described as altered in different tumor types. In EOC,

overexpression of *HOTAIR* was associated with poor prognosis and pro-metastatic effects, which are at least partially exerted by regulating the expression of metalloproteinases and EMT-related genes [22]. Recent reviews have summarized the role of lncRNAs in tumor progression and invasion [17,23].

High-throughput transcriptome studies have identified differential expression patterns of lncRNAs associated with disease recurrence and platinum resistance [24–26]. Furthermore, several aberrantly expressed lncRNAs in resistant OC cells have been functionally related to resistance mechanisms, including increased cell division, improved DNA repair, upregulation of drug transporters, or reduced susceptibility to apoptotic stimuli [17,27–29].

A well-known mechanism of transcriptional regulation is DNA methylation. The direct interaction between lncRNAs and proteins of the epigenetic machinery, such as chromatin modifiers and DNA methyltransferases, has emerged as an additional link between lncRNAs and tumorigenesis. Thus, knowledge of the interplay between lncRNAs with epigenetic modification and transcriptional regulation significantly contributes to understanding the mechanisms involved in gene regulation and unravels robust markers associated with resistance to therapy. Importantly, lncRNA targeting is one of the only therapeutic approaches that can upregulate tumor suppressor genes endogenously in a locus-specific manner [30]. Despite these promising findings and the large number of lncRNAs in the human genome, only a few of them have been well characterized, annotated, and explored. It is critical to elucidate the actual functions of cancer-related lncRNAs and their association with therapy resistance in OC. Moreover, the functional relationship between lncRNA expression and epigenetic regulation is not yet well-understood.

Several studies have demonstrated that lncRNAs are associated with tumor progression and chemotherapy resistance. However, the implications of DNA methylation changes, lncRNAs, and their association with chemotherapy response in OC are poorly explored. Herein, using RNA sequencing (RNA-seq) and DNA methylation analyses in OC cell lines, we identified the lncRNA *SNHG12* (small nucleolar RNA host gene 12) as a candidate biomarker of response to carboplatin in OC and investigated its functional mechanisms.

2. Materials and Methods

2.1. Cell Lines and Patient Samples

A panel of six OC cell lines (IGROV1, OVCAR3, OVCAR4, OVCAR5, OVCAR8, and Ovc316) were included in this study. The cell lines OVCAR3, OVCAR5, and Ovc316 were kindly provided by Robert Strauss, Danish Cancer Society, Denmark, and the cell lines IGROV1, OVCAR4, and OVCAR8 were donated by Wan Lam, University of British Columbia, Vancouver, Canada. The cell lines were cultured in RPMI medium (Sigma Aldrich, Saint Louis, MO, USA) (cell lines IGROV1, OVCAR3, and OVCAR4) or Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich) (OVCAR5, OVCAR8, and Ovc316), as recommended. The media were supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit HaEmek, Israel), and 1% antibiotic-antimycotic (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 µg/mL of Amphotericin B—Thermo Fisher, Waltham, MA, USA). After confluence, cells were treated with 0.05% trypsin/0.02% EDTA (Sigma Aldrich) and replicated. We tested cell cultures for mycoplasma contamination using a PCR-based assay once per month. The mutation profile of cancer-related genes described in these cell lines was confirmed by Sanger sequencing (Table S1).

High-grade serous ovarian tumor tissue samples ($n = 39$, FIGO stages III–IV) were obtained from patients who underwent surgical resection (mean age 69.46 ± 9.30 , ranging from 46 to 83 years old) at Horsens Hospital, DK, between 2005 and 2007. Follow-up and progression-free survival (PFS) data were retrieved from the Danish Health Registries and research databases of the Department of Oncology, University Hospital of Southern Denmark, Vejle, DK. The samples were classified according to the World Health Organization (WHO) classification of female genital tumors [31]. Recurrence within 12 months after the last platinum treatment was defined as platinum resistance. All patients were included at the time of primary diagnosis and followed after first-line treatment. PFS was defined

as the interval between the date of surgery and the date of disease progression or death by any cause. Overall survival (OS) was defined as the interval between the date of the treatment and death by any cause. In addition to tumor samples, eight normal ovarian tissues were obtained from individuals submitted to surgery for other causes than cancer and used as controls in the RT-qPCR experiments. The Table S2 summarizes the clinical data of the patients included in our study.

The study was conducted in accordance with the Declaration of Helsinki ethical guidelines and approved by the National Ethics Committee (case no. #1803521) and the Danish Data Protection Agency (19/48213). Informed consent was obtained from all patients prior to sample collection.

2.2. Establishment of Resistant Cell Lines

Carboplatin-sensitive cells (IGROV1, OVCAR3, OVCAR4, and OVCAR5) (Table S1) were exposed to increasing concentrations of carboplatin following an intermittent schedule with phases of recovery between the treatments. The baseline schedule consisted of 4 days of treatment with 1 μ M of carboplatin in the culture media followed by 3 recovery days in media without carboplatin. For cell lines tolerating this treatment well, the concentration was increased gradually (1, -5 , -10 , or -15 μ M) when the cells reached high confluency. For cell lines not tolerating the treatment well, the recovery was extended, and the low dose was maintained. The exact treatment regimen was dependent on the growth characteristics and drug response of the individual cell lines. In general, resistance was developed following 2–4 months of treatment. Acquired resistance was confirmed by IC₅₀ measurements before and after this period using a crystal violet assay for determining the viability of cultured cells as previously described [32,33].

2.3. RNA Extraction and Sequencing

Total RNA was isolated with the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The RNA purity and quantity were measured with a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity was assessed with RNA screen tape on a 2200 TapeStation (Agilent, Santa Clara, CA, USA).

Libraries were generated using 500 ng of total RNA and prepared using the TruSeq Stranded Total RNA Library Prep Gold kit (Illumina, San Diego, CA, USA), following the manufacturer's protocol. Integrated DNA Technology (IDT) for TruSeq RNA UD Indexes was added for multiplexing, and samples were paired-end sequenced with 2×100 bp on the NovaSeq 6000 system (Illumina) using the S1 Reagent Kit v1.5 (200 cycles), as recommended by the supplier. RNA-seq data quality control was performed using FastQC and MultiQC [34,35]. Reads were aligned using STAR (v.2.7.6a) [36], and the gene read count was performed with HTSeq-count using the Ensembl human genome assembly GRCh38, release 99. The final count matrix consisted of 60,676 genes (raw counts), including those with no counts. The differential expression analysis between carboplatin-sensitive and carboplatin-resistant cell lines was performed in the Galaxy bioinformatics platform using the default settings of the EdgeR package (including normalization TMM) [37,38]. The transcriptome data are deposited in the Gene Expression Omnibus (GEO) database (GSE198077).

2.4. Pathway Enrichment Analysis

We performed enrichment analysis using our list of DEGs to identify overrepresented pathways unique to sensitive and resistant OC cell lines. The analysis was performed using the Kyoto Encyclopedia of Genes and Genomes 2021 Human database via the Enrichr analysis tool [39].

2.5. DNA Extraction and DNA Methylation Profiling

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). A total of 210 ng of DNA was sodium bisulfite converted with the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's recommendations. The genome-wide methylation status of each sample was assessed using the Infinium MethylationEPIC 850 K BeadChip (Illumina, USA). The DNA methylation data is available at the GEO database (GSE198077). The microarrays were scanned using the Illumina HiScan system, and the data were processed using R language and analyzed as previously described [40]. For methylation data analysis, fluorescence intensity data (.IDAT) files were analyzed using the minfi R package [41]. Quality control, the p -value for all probes, background noise detection, and adjustment were performed using the same package. Differences Type-I and Type-II probes adjustment and normalization of β values were performed using the Beta Mixture Quantile dilation (BMIQ) model [42]. Batch effects were removed using the package SVA [43]. Probes with low quality (detection p -value > 0.05), mapped in X/Y chromosomes, and cross-reactive were filtered out, and those mapped to SNPs were removed (package minfi) [41,44]. Differentially methylated CpG positions (DMPs) were identified using the R package limma [45]. Probes with p -value < 0.01 and $|\Delta\beta| > 0.1$ were considered significant and annotated using the Illumina manifest file.

2.6. RNA-seq and DNA Methylation Integrative Analysis

Only lncRNAs showing differential expression and DNA methylation were selected for further analysis. Pearson correlation test (r values with $p < 0.05$) was applied to identify probes and genes/lncRNAs with positive ($r+$) or negative ($r-$) correlation between DNA methylation and gene expression.

2.7. External Data from The Cancer Genome Atlas

External RNA-seq data from The Cancer Genome Atlas (TCGA) was used as a validation dataset. A total of 111 RNA-seq gene count files from patients with high-grade serous ovarian cancer (HGSC) were downloaded from the Genomic Data Commons Data Portal (TCGA-OV project). Only patients who received treatment with carboplatin were selected from this data set. The data files were created using a similar bioinformatics pipeline to the one applied in the analysis of our experimental data (STAR-HTseq count). Clinical information was collected from a published study [46]. Based on PFS, the patients were categorized into either sensitive (PFS > 12 months, $n = 69$) or resistant (PFS < 12 months, $n = 42$) to carboplatin. Data normalization and differential expression analysis between sensitive and resistant samples were performed in the Galaxy bioinformatics platform using EdgeR [37,38].

2.8. Criteria Used to Select lncRNA for Functional Assays

We selected lncRNAs overexpressed in resistant compared to sensitive cell lines. We filtered out lncRNAs not associated with differential DNA methylation and lncRNAs that were downregulated in the resistant OC samples from the TCGA cohort. Among the remaining lncRNAs with the highest expression, we investigated their roles in cancer and response to therapy available in the literature. The final candidate, *SNHG12*, was further investigated by determining its expression levels in the internal cohort of 39 HGSC samples. Next, siRNA experiments were performed in selected OC cell lines. Bioinformatics analyses were also carried out using online lncRNA databases for investigating *SNHG12* subcellular localization (LncACT.db 3.0 available at <http://bio-bigdata.hrbmu.edu.cn/LncACTdb>, last accessed on 10 January 2022), regulatory relationships (LncRNADisease 2.0 available at <http://www.rnanut.net/lncrnadisease>, last accessed on 10 January, 2022), and co-expressed genes (Lnc2Cancer 3.0 available at <http://bio-bigdata.hrbmu.edu.cn/lnc2cancer/>, last accessed on 10 January 2022) [47–49].

2.9. RNA Extraction and RT-qPCR Assay

Total RNA was isolated from frozen ovarian tissues (39 tumor and 8 non-tumor samples) and OC cell lines using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription of 1 µg RNA was carried out with the High-Capacity RNA to cDNA kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative PCR was performed using TaqMan Fast Advanced Master Mix and TaqMan probes (assay ID: Hs00939627 for reference gene *GUSB* and assay ID: Hs00414754 for the target gene *SNHG12*). The RT-qPCR amplifications were performed in a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher, Waltham, MA, USA) with the following parameters: 50 °C for 2 min, 95 °C for 2 min, 40 cycles at 95 °C for 1 s, and 60 °C for 20 s. The relative expression in each sample was calculated using the $2^{-\Delta\Delta C_t}$ method [50].

2.10. Knockdown of lncRNA *SNHG12* and Carboplatin Exposure

Four OC cell lines (IGROV1 and its carboplatin-resistant counterpart IGROV1-R1, OVCAR8, and Ovc316) were selected for knockdown experiments of the lncRNA *SNHG12* using a small interference RNA (siRNA) approach. Cells were seeded into flat-bottom 6-well (3×10^5 cells/well) and 96-well (1×10^4 cells/well) tissue culture plates and maintained at 37 °C under 5% CO₂. Transfections were carried out with ~60% confluency in each of the four cell lines using Opti-MEM I Reduced Serum Medium, GlutaMAX Supplement, and Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's recommendations. The Silencer Select Negative Control siRNA #1 (cat# 4390843) and *SNHG12* siRNA (cat#4392422, ID: n543460) were used at 1 pmol (96-well plate) and 25 pmol (6-well plate) per well, respectively (Thermo Fisher, Waltham, MA, USA). Transfection efficiency was determined using BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Thermo Fisher, Waltham, MA, USA). After 48 h incubation, the medium was replaced with the growth medium only or medium containing two different concentrations of carboplatin (10 µM or 50 µM). Triplicates of each condition were analyzed in two independent experiments. The step-by-step workflow used to evaluate the *SNHG12* silencing is shown in Figure S1.

2.11. Cell Viability Assays

Following carboplatin treatment, cell viability was measured using the standard protocol of CellTiter Blue Viability Assay (Promega, Madison, WI, USA) at 24 h, 72 h, and 96 h time points. Fluorescence was recorded at 530/590 nm using the Synergy HT plate reader (BioTek, Winooski, VT, USA). Reading corrections were performed using wells with no cells treated under the same conditions.

2.12. Data Processing and Statistical Analyses

Data were generated from at least two independent experiments with triplicates. Graphical and statistical analyses were performed using Graph Pad Prism 9.0 (Graph Pad Software, San Diego, CA, USA) and Galaxy bioinformatics platform [37]. Clusters were generated using Euclidean distance and complete linkage. Group data are reported as mean ± SD. Analysis of variance (ANOVA with Tukey's multiple comparisons test and Fisher's exact test) was used to compare cell viability among the different conditions (siRNA versus scrambled RNA) and time points (24 h, 72 h, and 96 h). Survival analyses were performed using Kaplan–Meier and log-rank methods. Statistical significance was considered with p -values ≤ 0.05.

3. Results

3.1. Carboplatin Response in Ovarian Cancer Cell Lines and Establishment of Carboplatin-Resistant Derived Subpopulations

Among the six OC cell lines, four were classified as carboplatin sensitive (IGROV1, OVCAR3, OVCAR4, and OVCAR5), and two (OVCAR8 and Ovc316) were inherently resistant (IC₅₀ values above 32 µM). Carboplatin acquired resistance was observed in derived

subpopulations from IGROV1 (IGROV1-R1), OVCAR3 (OVCAR3-R1 and OVCAR3-R2), and OVCAR5 (OVCAR5-R1). OVCAR4 presented high sensitivity to carboplatin and, therefore, this cell line was not used in the functional assays. The resistance to carboplatin was validated by an increase in the IC_{50} from 4–17 μ M up to 28–44 μ M (Table S1 and Figure S2A). Two resistant subpopulations were derived in parallel from OVCAR3 (OVCAR3-R1 and OVCAR3-R2) and exhibited a right shift of the dose-response curves of carboplatin (Figure S2B). OVCAR3-R1 and OVCAR3-R2 were established from independent cultures.

3.2. Differential Expression Profiles Associated with Carboplatin Resistance in Ovarian Cancer Cell Lines and Ovarian Cancer Tissues

To assess differential gene expression between the sensitive and resistant OC cells, RNA sequencing was performed in the four cell lines with acquired in vitro resistance to carboplatin (IGROV1-R1, OVCAR3-R1, OVCAR3-R2, and OVCAR5-R1), four sensitive cell lines (IGROV1, OVCAR3, OVCAR4, and OVCAR5), and the two inherently carboplatin-resistant OC cell lines (Ovc316 and OVCAR8). Genes differentially expressed were determined by comparing the sensitive with the resistant cell lines. This comparison revealed 4255 differentially expressed genes (DEGs; $p < 0.05$), among which 1913 were upregulated and 2342 downregulated in carboplatin-resistant OC cells. The normalized read counts considering $FDR < 0.05$ and $\log FC < -1.5 / > 1.5$ revealed 86 differentially expressed genes comparing cell lines sensitive and resistant to carboplatin. Supervised clustering analysis of the significant genes between resistant and sensitive OC cells is depicted in Figure 1A. The two resistant subpopulations derived from OVCAR3, OVCAR3-R1 and R2, exhibited very similar transcriptional profiles.

The pathway enrichment analysis using the most significant genes with fold change > 1.5 between sensitive and resistant cells revealed 25 pathways upregulated and 117 downregulated in resistant cases ($p < 0.05$). Interestingly, several of the downregulated genes were involved in epigenetic regulation of gene expression, including DNA methylation and histone post-translational modifications (Table S3).

To select the DEGs that were also representative of resistance to carboplatin in a cohort of 111 HGSC patients from TCGA, we used differential expression analysis of carboplatin-sensitive ($n = 69$) and -resistant ($n = 42$) patients, identifying 8246 DEGs ($p < 0.05$) (Figure 1C). The comparison of DEGs between OC cell lines and OC primary tissues from the TCGA dataset resulted in 570 genes altered in both cohorts (Figure 1C), with 304 showing the same direction. Among those, 110 upregulated and 194 downregulated genes were associated with carboplatin resistance. Among the 304 DEGs overlapped between resistant cell lines and resistant OC patients from the TCGA dataset, 50 transcripts were categorized as lncRNAs, with 34 upregulated and 16 downregulated transcripts.

3.3. Differential Methylation Profile Associated with Carboplatin Resistance in Ovarian Cancer Cell Lines

Global DNA methylation was determined with the Infinium MethylationEPIC 850K array (Illumina) for the ten cell lines subjected to RNA sequencing. The comparison of resistant and sensitive cell lines revealed 14,529 differentially methylated CpG positions (DMPs, $p < 0.05$), 13,023 hypermethylated and 1506 hypomethylated in resistant OC cells. Clustering of the most significant DMPs (5721 positions with $p \leq 0.01$ and $|\Delta\beta| \geq 0.1$) clearly showed that resistant and sensitive cells have a distinct methylation profile (Figure 1B).

3.4. Integrative Data Analysis

The transcriptome and DNA methylation data integration unveiled 11 differentially expressed and methylated lncRNAs (Figure 1C) associated with 18 DMPs (Table S4), including the lncRNA *SNHG12*. The lncRNA *SNHG12* was selected for further analysis due to its increased expression in HGSC-TCGA patients (Figure 1D) and in resistant OC cell lines (Figure 1F,G). The cell lines OVCAR3-R1, OVCAR3-R2, IGROV1-R1, and OVCAR5-R1 showed increased *SNHG12* expression compared to their carboplatin-sensitive

counterparts (Figure 1G). Additionally, this lncRNA was associated with a hypomethylated CpG in carboplatin-resistant cell lines (Figure 1H), showing a negative correlation with *SNHG12* expression levels. Figure 2A,B depict the difference in expression levels and beta values of the ten lncRNAs and DMPs, respectively, according to sensitivity to carboplatin in OC cell lines. Six of these lncRNAs were upregulated in resistant cells, as shown in Figure 2A. The downregulated lncRNAs, including *EMX2OS*, presented hypermethylated CpG positions (Figure 2B)

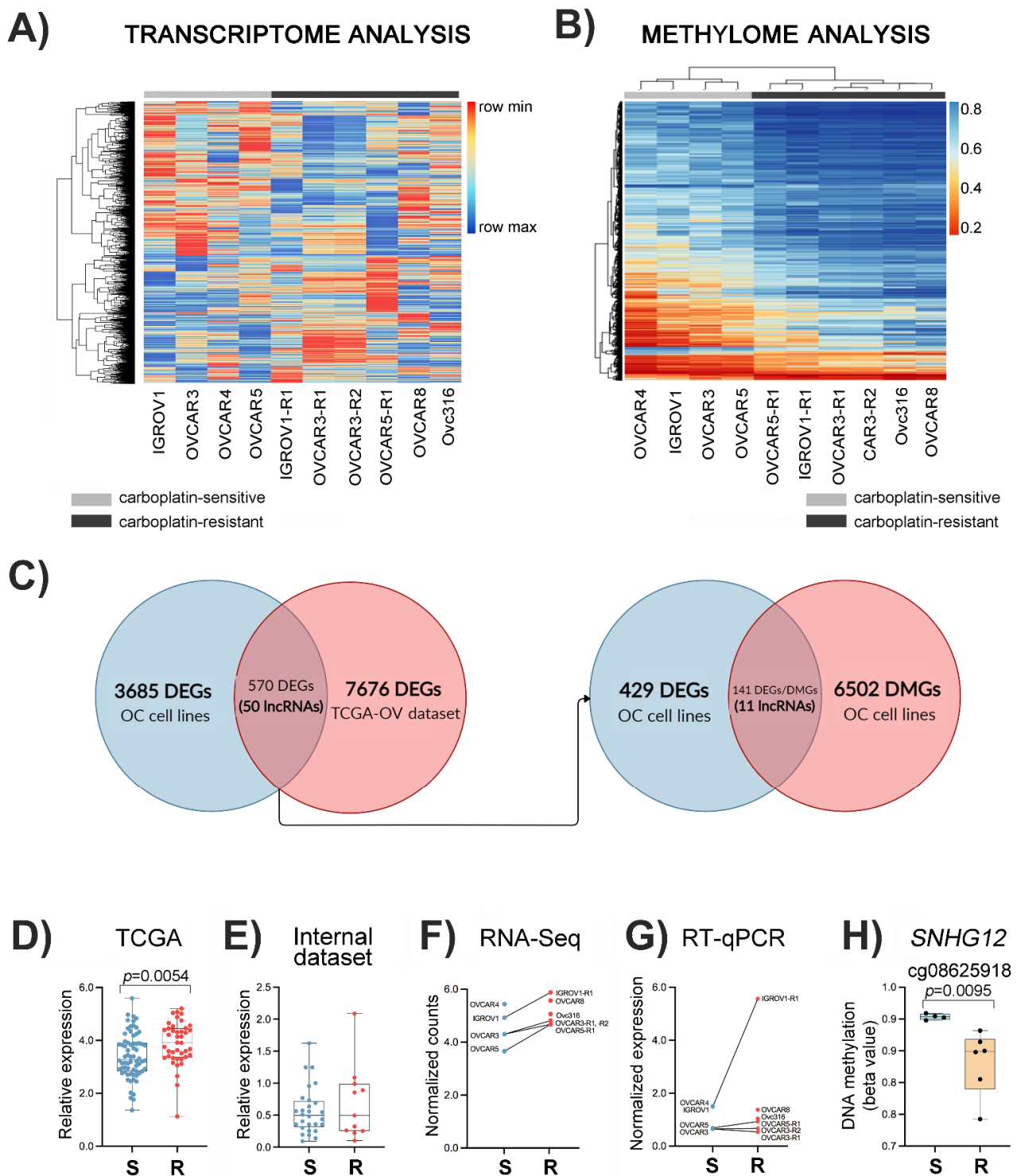


Figure 1. Identification of differentially expressed and methylated lncRNAs associated with carboplatin resistance in ovarian cancer (OC) cell lines. (A) Clustering analysis with normalized read counts

of 4255 significantly differentially expressed genes (DEGs) in cell lines sensitive and resistant to carboplatin. (B) Heatmap with the 5721 DMPs obtained after comparing the resistant and sensitive cell lines. (C) The Venn diagram shows the intersection between DEGs detected in the ten OC cell lines and TCGA-OV dataset when comparing carboplatin-sensitive and -resistant specimens, including 50 lncRNAs. Integrative transcriptome and methylome analysis revealed that 11 lncRNAs were DEGs and differentially methylated genes (DMGs). The expression levels of the lncRNA *SNHG12* in OC tissues and cell lines, classified as sensitive or resistant to chemotherapy, are shown at the bottom. (D) The first graph presents data retrieved from TCGA-OV ($n = 111$ cases, 69 sensitive and 42 resistant), and (E) the second was obtained from an internal cohort analyzed by RT-qPCR ($n = 39$ cases, 28 sensitive and 11 resistant). The comparison of lncRNA *SNHG12* expression levels determined by RNA-Seq and RT-qPCR analysis in OC cell lines and their carboplatin-resistant counterparts are shown in (F,G), respectively. (H) DNA methylation of cg08625918 (mapped on *SNHG12* locus) in carboplatin-sensitive and -resistant OC cell lines. (S, carboplatin-sensitive and R, carboplatin-resistant OC cell lines.)

3.5. Platinum Resistance-Associated Genes

The list of 4255 DEGs and 14,529 DMPs detected in our panel of OC cell lines was compared with the platinum response-related gene list (937 genes/proteins) recently curated by Huang et al. [51]. We found 254 DEGs and 142 DMPs overlapping with the database entries (Table S5).

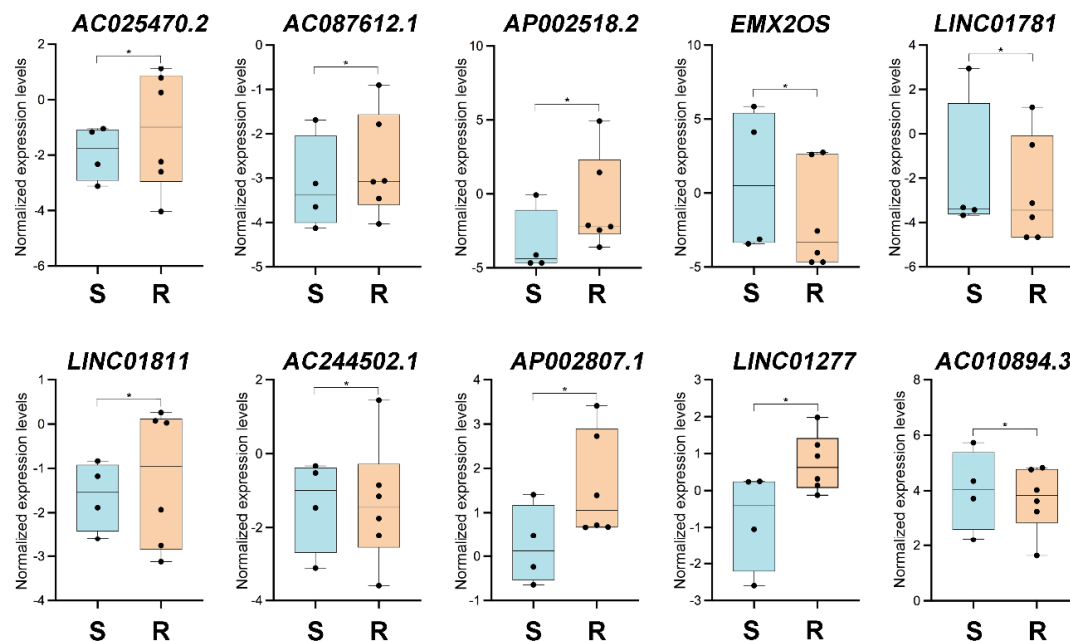
3.6. The lncRNA *SNHG12* Is Overexpressed in Ovarian Cancer

The expression of the lncRNA *SNHG12* was also quantified in an independent cohort of 39 HGSC and 7 normal ovarian tissues (internal dataset). We found no significant differences between patients sensitive and resistant to carboplatin, possibly due to the limited number of resistant patients (Figure 1C). To evaluate the clinical significance of *SNHG12* expression in OC patients, we divided the cohort into *SNHG12* high expression and low expression groups according to the cutoff value, which was defined as quartiles. As demonstrated in Figure S3, patients from HGSC-TCGA with higher expression of *SNHG12* presented shorter PFS ($p = 0.03$). However, no significant difference was observed for OS in this cohort and for PFS (6 or 12 months) and OS in our internal dataset.

3.7. Functional Analysis of the lncRNA *SNHG12*

We investigated the function of the lncRNA *SNHG12* on the carboplatin resistance in OC cells. The inherently resistant Ovc316 and OVCAR8 and the pair IGROV1 (sensitive) and IGROV1-R1 (IGROV1-derived carboplatin-resistant subpopulation) were transfected with si-*SNHG12*. Carboplatin dose-response curves were confirmed by cell viability assay before knockdown experiments (Figure 3A–D). The RT-qPCR results showed down-regulation of *SNHG12* in all cells transfected with siRNA-*SNHG12*. *SNHG12* inhibition increased the sensitivity to carboplatin of IGROV1, Ovc316, and OVCAR8 cells as indicated by reduced viability after 96 h of exposure to 50 μ M carboplatin in the siRNA transfected cells (Figure 3A–D). This effect was also observed in the Ovc316 cell line after 72 h of drug exposure (Figure 3C). IGROV1-R1 seemed to have a more pronounced effect of *SNHG12* inhibition on carboplatin sensitivity after 72 h than 96 h, although not significant. Exposure to 10 μ M carboplatin was not enough to trigger the combined effect with *SNHG12* inhibition on cellular viability. These results indicated that OC cells maintaining a high *SNHG12* expression are more resistant to carboplatin. Therefore, this lncRNA is probably associated with the mechanism of carboplatin resistance in OC cell lines. The effect of siRNA-*SNHG12* was not observed in the IGROV1-R1 cell line at 50 μ M of carboplatin exposure (Figure 3B).

A)



B)

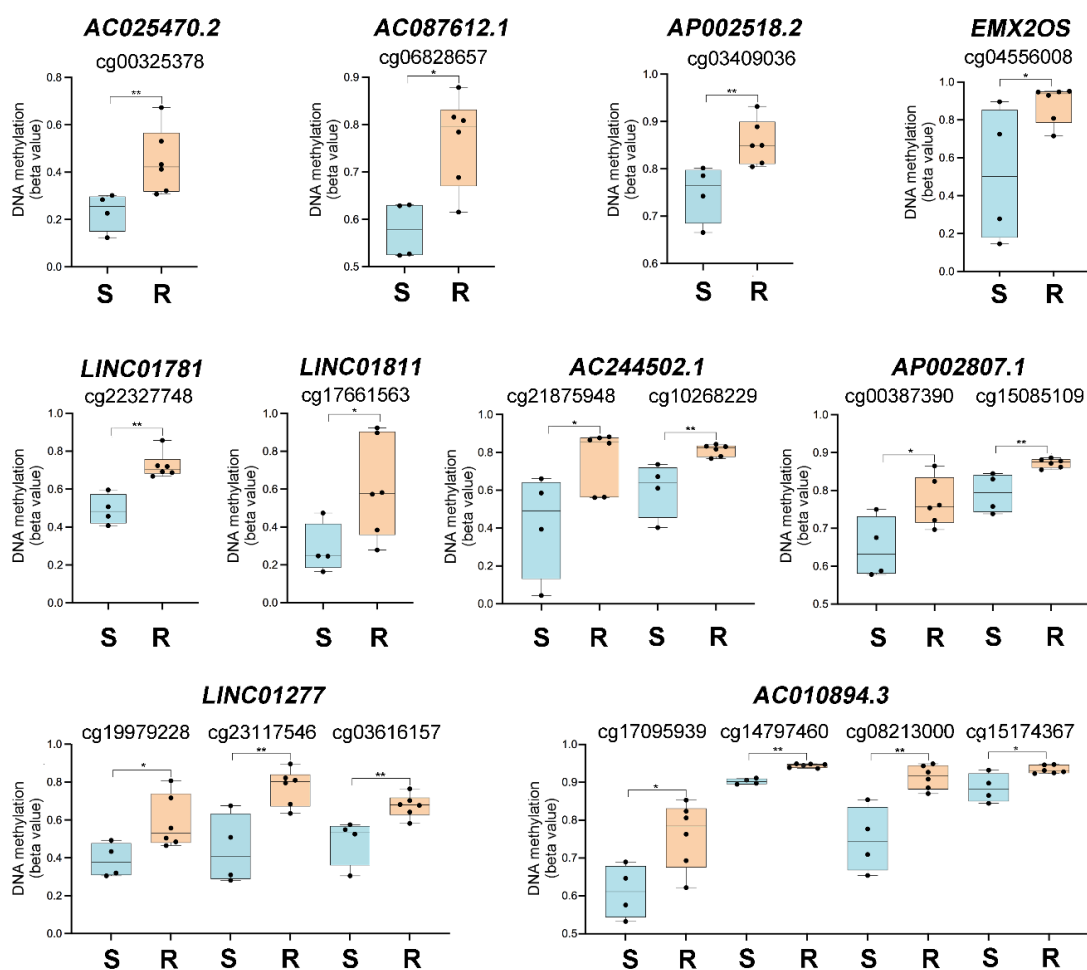


Figure 2. lncRNAs associated with carboplatin resistance in ovarian cancer (OC) cell lines. (A) Expression levels of ten out of eleven selected long non-coding RNAs were detected as differentially

expressed and methylated in OC cell lines according to sensitivity or resistance to carboplatin. (B) Comparison of DNA methylation levels (beta value) of differentially methylated positions (CpG) located nearby to the ten selected long non-coding RNAs in sensitive and resistant cell lines. (*) $p < .005$ and (**) $p < 0.01$ (non-parametric t test). (S, carboplatin-sensitive and R, carboplatin-resistant OC cell lines).

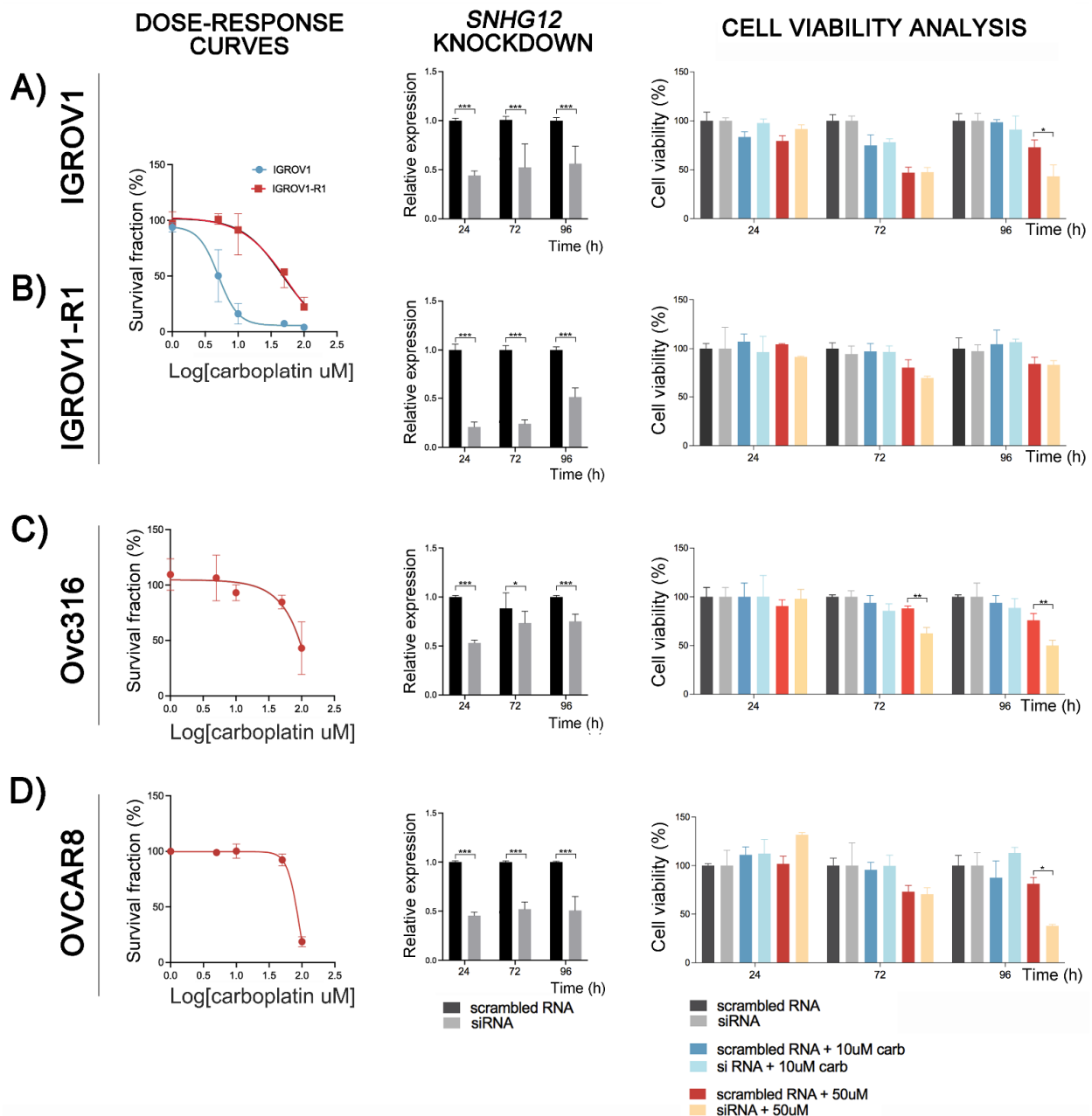


Figure 3. Effect of *SNHG12* knockdown on the cell viability of ovarian cancer (OC) cell lines exposed to carboplatin. Dose-response curves of carboplatin treatment, the efficiency of the siRNA targeting the lncRNA *SNHG12*, and its effects on cell viability after carboplatin treatment are shown for the IGROV1 and its induced resistant-counterpart IGROV1-R1 (A,B), and in intrinsically resistant Ovc316 (C) and OVCAR8 (D), respectively. The cells were exposed to scrambled RNA or *SNHG12* siRNA and treated with carboplatin (10 μ M or 50 μ M) during 24, 72 and 96 h. Cytotoxicity was determined by the CellTiter Blue Viability Assay. Knockdown led to a reduction of the relative expression levels of the lncRNA *SNHG12* compared with the scrambled RNA after 24, 72 and 96 h in the IGROV1/IGROV1-R1 (A,B), Ovc316 (C), and OVCAR8 (D) cells. (*) $p < 0.05$; (**) $p < 0.001$; (***) $p < 0.0001$.

4. Discussion

A repertoire of molecular and functional studies in OC has been useful for tumor stratification predicting different clinical outcomes [10,52–54]. DNA methylation profiling allowed the identification of three molecular subgroups with significant survival differences in HGSCs [55]. DNA methylation of specific genes has shown potential for monitoring disease progression and response to treatment [56]. Methylation of the *BRCA1* promoter is recognized as a somatic driver in approximately 11% of HGSCs, and its loss was associated with acquired chemoresistance [10,52]. In a platinum-sensitive OC cohort mostly comprised of patients with HGSC, the combination of *CAMK2N1* (calcium/calmodulin dependent protein kinase II inhibitor 1) and *RUNX3* (RUNX family transcription factor 3) methylation in tumor specimens was associated with shorter OS and PFS [57]. Additionally, dysregulated DNA methylation may interfere with the expression of lncRNAs in OC, disrupting many processes, including tumor suppressive and oncogenic activities [58–60].

In this study, we evaluated the interplay between the DNA methylome and transcriptome profiles in carboplatin-resistant OC cell lines and validated our findings with functional assays and gene expression in OC tissues from HGSC patients. We focused on identifying lncRNA genes both differentially expressed and affected by methylation changes associated with the development of platinum-resistance in OC. An integrative analysis using an external cross-validation set of selected OC samples from TCGA revealed 570 DEGs, including 50 lncRNAs. In this last group, 11 lncRNAs were also associated with at least 1 DMP. Among these 11 lncRNAs, *SNHG12*, *EMX2OS* (*EMX2* opposite strand/antisense RNA), and *AC010894.3* had their roles previously investigated in OC, but the mechanisms involved in the transcriptional regulation were barely explored [61–64]. Our DNA methylation analysis showed that *EMX2OS* exhibited a trend towards hypermethylation in resistant OC cells, while *AC010894.3* was hypermethylated in the same cell lines. *EMX2OS* was previously reported as upregulated in OC tissues and cell lines, enhancing proliferation, invasion, and sphere formation in vitro and tumor growth in vivo [63]. Based on a risk score model, *AC010894.3* was included in a four-lncRNA signature that accurately predicted the OS of OC patients, and its increased expression was associated with better survival [62]. Three other lncRNAs from our gene list, *AC244502.1*, *AP002807.1*, and *AP002518.2*, were integrated into prognostic signatures for breast cancer, clear cell renal cell carcinoma, and Wilms tumor, respectively [65–67]. The lncRNA *SNHG12* was selected as a candidate due to its inverse correlation between high expression levels and DNA hypomethylation in carboplatin-resistant OC cell lines.

SNHG12 is a lincRNA (long intergenic non-coding RNA) and a member of non-coding genes that host small nucleolar RNAs (snoRNA). Approximately 30 *SNHG* genes were identified in the human genome, and some of them (*SNHG1*, *SNHG3*, *SNHG5*, *SNHG6*, *SNHG7*, *SNHG12*, *SNHG15*, *SNHG16*, and *SNHG20*) have been reported as overexpressed in different types of human cancers [68]. Differently from snoRNAs, which arise exclusively from introns and are classified as small ncRNAs, the transcripts from *SNHG* genes are processed and contain exons. Additionally, *SNHG* RNAs differ from snoRNAs by their subcellular location both in the nucleus and the cytoplasm. Specifically, interactions between *SNHG12* and microRNAs have been implicated in several cancer-related cellular processes and signaling pathways. Experimental analysis showed that *SNHG12* is an endogenous sponge for microRNAs in cervical, prostate, gastric, breast, lung, colorectal cancer, gliomas, osteosarcoma, hepatocellular, papillary thyroid, and clear cell renal cell carcinomas (reviewed in [68,69]). The diversity of interactions with microRNAs showcased the complex molecular network influenced by *SNHG12* and indicated that it might serve as a new therapeutic target in human cancers.

Significantly higher expression levels of *SNHG12* were described in hepatocellular, papillary thyroid, and cervical carcinomas compared to normal tissues, among other cancer types [70–73]. Association of *SNHG12* expression level with advanced FIGO stage, vascular involvement, and lymph node metastasis in cervical cancers evidenced its potential clinical significance [70]. *SNHG12* overexpression predicted poor prognosis in prostate

cancer [74,75], while it was associated with tumor progression and poor survival rates in gastric and lung cancer [76,77]. In our internal dataset, HGSC tissues ($n = 39$) showed higher *SNHG12* expression levels compared to normal ovarian samples ($n = 5$); however, no statistical significance was detected with histopathological and clinical parameters, probably due to the limited number of cases. Sun and Fan evaluated the expression levels of *SNHG12* by RT-qPCR in 24 matched OC and adjacent normal tissues and reported that *SNHG12* levels were higher for patients with stage III-IV than for those with stage I-II, as well as for metastatic patients [61]. Additionally, the 5-year survival analysis was worse in OC patients with high-level relative to those with low-level *SNHG12* expression. Using the OC dataset from TCGA, we found high *SNHG12* expression levels associated with decreased PFS (Figure S3). Altogether, these findings indicate that *SNHG12* is a biomarker of poor prognosis in OC.

We used a siRNA strategy to inhibit *SNHG12* and verified the reduced cell viability of resistant OC cell lines exposed to carboplatin treatment. A previous study detected that A2780 and HO8910 OC cell lines overexpressed *SNHG12* while miR-129 was repressed [61]. The authors demonstrated that *SNHG12* promotes the proliferative and migratory abilities of those OC cells by sponging the miRNA-129 and releasing its target, the *SOX4* (SRY-box transcription factor 4) mRNA. In vitro studies also showed the effect of *SNHG12* repression in inhibiting proliferation, migration and invasion, and metastasis in osteosarcomas and gastric cancer [78,79]. The suppression of *SNHG12* also induced apoptosis and cell cycle arrest, while the opposite effects were observed after the forced expression of *SNHG12* [63]. Similar findings were reported in breast cancer [80]. Of note, *SNHG12* is a downstream target gene of the oncoprotein MYC and its upregulation mediates cell proliferation and migration in triple-negative breast cancer [81].

The mechanism by which *SNHG12* mediates the multi-drug chemoresistance is yet unclear. However, it is recognized that the *SNHG12* knockdown can reverse the resistance to cisplatin, paclitaxel, and gefitinib in cell lines derived from lung cancer [77]. *SNHG12* mediated doxorubicin resistance in osteosarcoma, while its knockdown contributed to the upregulation of miR-320a and improved the sensitivity to this drug [82]. In gliomas, hypomethylation of the promoter region of *SNHG12* was correlated with the transcription factor SP1, leading to its up-regulation in temozolomide-resistant cell lines [83]. In renal cell carcinomas, *SNHG12* was involved in the regulation of the *CDCA3* (cell division cycle associated 3) gene mediated by SP1, promoting resistance to sunitinib [84]. These data indicate that *SNHG12* dysregulation is involved in tumorigenesis, impacts diverse cellular processes, and promotes resistance to chemotherapy.

In addition to the cytoplasm, *SNHG12* was also detected in the nucleus, nucleoplasm, nucleolus, and chromatin [47]. Importantly, regulatory relationships were predicted in a genome region spanning 350Kb that flanks the *SNHG12* locus and includes six genes mapped upstream (*PHACTR4*, *RCC1*, *TRNAU1AP*) and downstream (*TAF12*, *RAB42*, and *GMEB1*) [48]. *SNHG12* expression correlates with genes mapped within this region in the 111 OC samples. Our RNA-seq data showed that while the *SNHG12* was upregulated in carboplatin-resistant OC cell lines, the neighboring genes (*PHACTR4*, *RCC1*, *TAF12*, and *GMEB1*) were downregulated in those cells (Figure S4). This finding indicates that *SNHG12* may act as a guide of transcription factors and other protein complexes, such as those involved in cis-regulatory epigenetic mechanisms. This hypothesis is further supported by experimental evidence curated in bioinformatic tools. Co-expression networks demonstrate that *SNHG12* is correlated with genes encoding transcriptional activators such as *SP1* (Sp1 transcription factor), *MYC* (MYC proto-oncogene, bHLH transcription factor), and *STAT2* (signal transducer and activator of transcription 2) in OC and predict interactions between *SNHG12* and HDAC9 protein by Cross-Linking and ImmunoPrecipitation (CLIP) experiments [49,85,86]. The *HDAC9* (histone deacetylase 9) gene is overexpressed in cancer cells and is a member of a family of enzymes responsible for the deacetylation of lysine residues, a key event in the aberrant epigenetic repression in cancer [87]. Other *SNHG* genes, including *DANCR* (former *SNHG13*), *SNHG14*, and *SNHG15*, act synergistically with *EZH2*

(a histone methylase and catalytic subunit of the Polycomb Repressive Complex 2) to inhibit the expression of downstream target genes in endocrine-related cancers [69]. Recently, Huang et al. surveyed the literature on platinum resistance published in the last 30 years and compiled a list of 937 platinum response-associated genes/proteins. Comparing this set of genes with our RNA-seq and DNA methylation results, we verified that 254 DEGs and 142 DMPs overlapped with entries on the list [51]. The genes encoding *HDAC1* (histone deacetylase 1) and DNA demethylase *TET1* (tet methylcytosine dioxygenase 1) were DEGs, and the *EZH2* (enhancer of zeste 2 polycomb repressive complex 2 subunit) gene associated with DMPs was also DEG in carboplatin-resistant cell lines. Previous studies reported that *EZH2* was generally overexpressed and implicated in the advanced stage, platinum resistance, and poor patient survival in OC [88]. Taken together, these data demonstrate an intrinsic connection between lncRNAs and disrupted epigenetic regulation of gene expression associated with carboplatin-resistance.

5. Conclusions

The integrative transcriptomic and epigenomic approach revealed that *SNHG12* is differentially expressed and methylated in OC cells. *SNHG12* modulates carboplatin-response in the cell lines tested, although the mechanism involved in this process should be deeper studied. The plethora of cancer-related features associated with the disrupted expression of *SNHG12* can be a result of the complex network interactions of this lncRNA with miRNAs and proteins. While DNA methylation changes explain the aberrant expression of *SNHG12* in cancer cells, emerging data indicate that *SNHG12* may control the expression of neighboring genes probably by changing epigenetic marks on histones and affecting their transcription.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers14071664/s1>, Figure S1: Workflow of the steps used to evaluate the effects of *SNHG12* knockdown and response to carboplatin in four ovarian cancer cell lines (IGROV1, IGROV1-R1, Ovc316, and OVCAR8); Figure S2: The establishment of carboplatin-resistant cell lines; Figure S3: Kaplan–Meier survival curves of progression-free survival (PFS) and overall survival (OS) according to the expression level of the lncRNA *SNHG12* in ovarian cancer patients; Figure S4: Differential expression of genes in the *SNHG12* locus and encoding components of the epigenetic machinery; Table S1: Mutational status of four cancer driver genes (*TP53*, *BRCA1*, *BRAC2*, and *KRAS*) and carboplatin response of selected cell lines and its carboplatin-resistant counterparts; Table S2: Clinical and pathological features of 39 patients with high-grade serous ovarian carcinoma included in this study; Table S3: Pathway enrichment analysis of differentially expressed genes between carboplatin-sensitive and carboplatin resistant OC derived cells; Table S4: Differentially expressed lncRNAs and overlapping differentially methylated positions in ovarian cancer cell lines relative to its carboplatin-resistant counterparts; Table S5: Differentially expressed genes and differentially methylated positions associated with platinum response.

Author Contributions: Conceptualization, C.A. and S.R.R.; methodology, C.A., L.M.d.C. and C.A.R.; software, F.A.M.; validation, C.A., L.M.d.C. and C.A.R.; formal analysis, C.A., N.C. and F.A.M.; investigation, C.A., L.M.d.C. and C.A.R.; resources, S.R.R., K.D.S. and M.W.; data curation, C.A. and S.R.R.; writing—original draft preparation, C.A., C.A.R, L.M.d.C. and N.C.; writing—review and editing, S.R.R. and C.A.R.; visualization, C.A., C.A.R., L.M.d.C. and N.C.; supervision, S.R.R.; project administration, S.R.R.; funding acquisition, S.R.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the National Ethics Committee (case no. #1803521) and the Danish Data Protection Agency (19/48213).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE198077 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198077>, accessed on 10 January 2022).

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6. Capítulo 3

Este capítulo está relacionado com o manuscrito que será submetido ao periódico *Journal of Experimental & Clinical Cancer Research* (ISSN 1756-9966; Fator de impacto: 11,3):

Calanca, N.; Rainho, C.A.; Maeda, D.K.; Villacis, R.A.R; Bizinelli, D.; Waldstrøm, M.; Aagard, M.M.; Fokdal, L.; Steffensen, K.D.; Jensen, L.H.; Rogatto, S.R. Transcriptome analysis of organoids derived from malignant effusions of serous ovarian cancer patients reveals epigenetic regulators as potential therapeutic targets.

O manuscrito abordado neste capítulo representa o desfecho principal do projeto proposto no doutorado. A fim de retratar com maior fidedignidade o panorama transcricional do SOC, principalmente a expressão de lncRNAs, nós estabelecemos TDOs a partir de amostras de ascite e efusão pleural de 15 pacientes com SOC. Esses modelos experimentais são conhecidos por recapitularem o comportamento tumoral *ex vivo*, pois preservam a heterogeneidade histológica e molecular dos tumores de origem, diferentemente da cultura 2D⁸⁶. O transcriptoma dos TDOs e das amostras de tecido ovariano normal foi avaliado por RNA-Seq. A análise dos dados revelou um conjunto de lncRNAs diferencialmente expressos entre esses dois grupos, a maioria deles com níveis reduzidos nos TDOs. As linhagens OVCAR8 e Ovc316, caracterizadas como intrinsecamente resistentes à carboplatina no capítulo 2, foram utilizadas para testar algumas das hipóteses levantadas a partir dos dados em larga-escala obtidos com os organoides derivados de SOC. Em seguida ao pré-tratamento dessas células com as *epi-drugs* decitabina e tazemetostate, verificamos maiores níveis de expressão do lncRNA *MEG3* e maior sensibilidade à carboplatina. Esse transcrito foi associado à função de supressor tumoral e sua expressão encontra-se comumente reduzida ou perdida devido à

hipermetilação de seu promotor em diferentes tipos de câncer ^{87,88}. De fato, em nosso estudo nós observamos níveis menores de expressão de *MEG3* nos TDOs e amostras externas de SOC comparados com amostras de tecido ovariano normal. Organoides derivados de SOC de um caso em que havia células disponíveis foram submetidos ao regime de tratamento aplicado às linhagens celulares derivadas de OC e verificamos um aumento da sensibilidade à carboplatina mediante o uso de decitabina ou decitabina associada com tazemetostate. Estudos futuros devem expandir o número de TDOs expostos a esse tratamento para fornecer evidências mais robustas da eficácia das *epi-drugs* na restauração da sensibilidade à platina, como sugerimos.

**TRANSCRIPTOME ANALYSIS OF ORGANOID DERIVED FROM MALIGNANT
EFFUSIONS OF SEROUS OVARIAN CANCER PATIENTS REVEALS EPIGENETIC
REGULATORS AS POTENTIAL THERAPEUTIC TARGETS**

Naiade Calanca^{1,2}, Claudia Aparecida Rainho², Debora Kazumi Maeda^{1,2}, Rolando André Rios
Villacis^{1,3}, Daniela Bizinelli⁴, Marianne Waldstrøm⁵, Mads Malik Aagard¹, Lars Fokdal^{6,7},
Karina Dahl Steffensen^{6,7}, Lars Henrik Jensen^{6,7*}, Silvia Regina Rogatto^{1,7,8*}

¹Department of Clinical Genetics, University Hospital of Southern Denmark, Vejle, Denmark.

² Department of Chemical and Biological Sciences, Institute of Biosciences, São Paulo State
University (UNESP), Botucatu, SP, Brazil.

³ Department of Genetics and Morphology, University of Brasilia (UnB), Brasilia, DF, Brazil

⁴ Interunit Graduate Program in Bioinformatics, Institute of Mathematics and Statistics,
University of São Paulo (USP), São Paulo, SP, Brazil.

⁵ Department of Clinical Pathology, University Hospital of Southern Denmark, Vejle,
Denmark.

⁶Department of Oncology, University Hospital of Southern Denmark, Vejle, Denmark.

⁷ Institute of Regional Health Research, University of Southern Denmark, Odense, Denmark.

⁸ Botucatu Medical School Hospital, São Paulo State University – UNESP, Botucatu, SP,
Brazil

*Equal contribution

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27 **Corresponding author:**

28 Professor Silvia Regina Rogatto

29 Department of Clinical Genetics, University Hospital, Vejle

30 Institute of Regional Health Research, University of Southern Denmark

31 Beriderbakken 4, 7100 - Vejle – DK

32 Telephone number: +45 -2938 5610

33 Email: silvia.regina.rogatto@rsyd.dk and silvia.rogatto@unesp.br

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ABSTRACT

Background

Serous ovarian carcinoma (SOC) is an aggressive disease (80-90% of advanced high-grade tumors) characterized by resistance to standard-of-care platinum chemotherapy. Clinical trials demonstrated that epigenetic priming with hypomethylating agents resulted in clinical benefit in platinum-resistant OC patients. Herein, we characterized the expression of long noncoding RNAs (lncRNAs) and investigated their interplay with the epigenetic machinery in SOC-derived organoids. We also searched for targets actionable by epigenetic drugs that could improve SOC response to carboplatin.

Methods

Tumor-derived organoids (TDOs) were established from malignant effusions of 15 SOC patients. RNA sequencing (RNA-Seq) data from 18 TDOs and seven normal ovarian tissue samples was obtained and analyzed. We also retrieved external RNA-Seq data of SOC-derived organoids (N=4), SOC tissues (The Cancer Genome Atlas, N=337), and normal ovarian samples (The Genotype-Tissue Expression, N=180) to cross-validate our findings. We used public databases to filter differentially expressed genes for epigenetic regulators and predict interactions between lncRNAs and components of the epigenetic machinery. Next, we selected the epigenetic drugs decitabine (DAC) and tazemetostat (TAZ), whose targets were overexpressed in SOC, to treat two carboplatin-resistant OC cell lines and one TDO. Following the treatment with DAC and TAZ, the cell lines and the TDO were challenged with carboplatin.

Results

The comparison between our TDOs and normal samples revealed 1,428 lncRNAs and 5,241 protein-coding genes (PCGs) differentially expressed. Twelve lncRNAs and 168 PCGs potentially involved in epigenetic regulation were identified, including the lncRNA *MEG3* and

epigenetic effectors *DNMT3B* and *EZH2*. Their altered expression in SOC was confirmed in external datasets. The interaction network displayed *MEG3* with the highest centrality interacting with 20 predicted epigenetic effectors. The drug testing demonstrated that all cells showed increased sensitivity to carboplatin after DAC treatment, alone or in combination with TAZ, and the cell lines showed augmented *MEG3* expression.

Conclusions

Overall, our analyses unveiled epigenetic regulators abnormally expressed in SOC, such as *MEG3*, which are promising targets for overcoming chemoresistance.

Keywords: serous ovarian cancer, tumor-derived organoids, long non-coding RNAs, epigenetic drugs, *MEG3*.

BACKGROUND

Ovarian cancer (OC) is characterized by histological and molecular heterogeneity, which affects clinical outcomes (Blagden, 2015). Serous ovarian carcinoma (SOC), the most common and deadly histological type of OC, is an aggressive disease associated with relapse, resistance to chemotherapy, and poor prognosis (approximately 30% 5-year survival) (Gaitskell et al., 2022). SOC comprises high- and low-grade subtypes, in which high-grade is the most frequently diagnosed and responsible for 70-80% of deaths due to the disease (Lisio et al., 2019). Newly diagnosed OC cases are treated with chemotherapy regimens, usually combining platinum-based agents (cisplatin or carboplatin) with taxanes (e.g., paclitaxel) (Richardson et al., 2023). Although high-grade SOC (HGSOC) initially responds to chemotherapy, 80-90% of advanced-stage tumors are estimated to become resistant (Bowtell et al., 2015). The treatment of choice for low-grade SOC (LGSOC) is based on primary debulking surgery due to its lack of responsiveness to chemotherapy (Grisham et al., 2023).

Transcriptomic and epigenetic signatures (e.g., noncoding RNA expression, histone modifications, and DNA methylation) have been associated with clinical outcomes (Weberpals et al., 2011; Xiao et al., 2022). In OC tissues, epigenetic aberrations such as DNA hypermethylation and repressive histone marks were implicated in platinum resistance and responsible for silencing genes in pathways and processes associated with chemotherapy response (Moufarrij et al., 2019; Zhao et al., 2022). Thus, harnessing epigenetic changes is a promising strategy to overcome chemoresistance. Also, a combination of drugs targeting different molecular alterations might be more effective than the conventional approaches to treat this heterogeneous disease.

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that usually are not translated into proteins and can modulate gene expression by directly interacting

with proteins of the epigenetic machinery, such as histone modifying and chromatin remodeling complexes (Calanca et al., 2020; Mercer & Mattick, 2013; Xie et al., 2021). Aberrant lncRNA expression has been implicated in OC progression and response to therapy (Abildgaard et al., 2020; Wu et al., 2023). Recently, Chen et al. (2023) reported an OC prognostic risk model based on four lncRNAs whose expression was correlated with methylation regulators (L. Chen et al., 2023). The expression levels of five lncRNAs were positively associated with FIGO (International Federation of Gynaecology and Obstetrics) stage and lymph node metastasis, while other 62 lncRNAs were differentially expressed in cisplatin-resistant OC (Q. Li et al., 2018; H. Wang et al., 2016). As lncRNAs cooperate with epigenetic effectors in fine-tuning gene expression, the dysregulation of these transcripts may interfere with the course of OC by influencing dynamic epigenetic control.

Organoids are three-dimensional (3D) multicellular *in vitro* tissue constructs that recapitulate molecular factors and other aspects of their corresponding *in vivo* organ, capturing development, homeostasis, and pathogenesis complexity in more detail than 2D monolayer cultures (Clevers, 2016; Drost & Clevers, 2018). Tumor-derived organoids (TDOs) retain the tumor inter- and intra-patient heterogeneity and resemble the genomic and phenotypic features of the parental tumor, including response to therapy (Carvalho et al., 2022; de Witte et al., 2020; Qin et al., 2022). Malignant effusions from OC patients are a promising material for deriving TDOs since they contain tumor and non-tumor cells, such as fibroblasts, adipocytes, mesothelial, endothelial, and inflammatory cells (Ford et al., 2020; Sorolla et al., 2021). OC is the most common cancer type associated with ascites, which is present in more than one-third of patients, and the sixth cancer type causing pleural effusion (Kipps et al., 2013; Porcel et al., 2014). These malignant fluids are repeatedly drained during the disease course to alleviate symptoms, making them excellent material for studying the tumor progression and the resistance to therapy in SOC. Previous studies demonstrated the suitability of HGSOC ascites

for *in vitro* drug sensitivity testing and identifying biomarkers that can predict clinical resistance (den Ouden et al., 2020; Matte et al., 2019). A recent study used organoids derived from ascites of SOC patients to assess the potential of epigenetic drugs (epi-drugs) in improving the response to immunotherapy (Gerton et al., 2023). In the clinical setting, a few reports described the use of TDOs to personalize treatment selection for individual patients (W. Chen et al., 2023; Jensen et al., 2023).

In this study, we generated TDOs from malignant effusions to characterize the expression of lncRNAs and their interplay with the epigenetic machinery. Using transcriptome analysis and drug testing, we identified abnormally expressed epigenetic regulators that can be targeted with epigenetic drugs. Our experimental models have the potential to be translated into personalized drug screening platforms for SOC patients.

METHODS

Patients

Twenty-one serous ovarian carcinoma patients who presented malignant effusions (ascites or pleural effusion) and were referred to the University Hospital of Southern Denmark, Vejle, DK (February 2020 to June 2022) for paracentesis or thoracocentesis were eligible to participate in the study. All patients signed an informed consent form. Patients with clinically relevant medical conditions (e.g. uncontrolled hypertension and heart disease) that could impair the study results were excluded. Eight out of 21 SOC and seven normal ovarian samples were obtained from a previous study conducted by our group (Carvalho et al., 2022). The normal samples were collected from individuals who underwent surgery for other causes than cancer. For two SOC patients, more than one sample was collected. Clinical data was retrieved from

electronic medical records (Table 1). In the last follow-up (April 2024), all patients were dead due to the disease.

Tumor-derived organoids (TDOs) generation and culture

Malignant effusion fluids were collected from 21 SOC patients and processed immediately after drainage. TDOs were established following a protocol previously described by our group (Carvalho et al., 2022). Briefly, cells were washed in medium and resuspended on StemPro™ hESC SFM growth full medium (DMEM/F-12, GlutaMAX™, 25% bovine serum albumin, and StemPro® hESC Supplement) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented, at final concentration, with 8 ng/mL human FGF-basic (Thermo Fisher Scientific), 100 µM 2-mercaptoethanol (Thermo Fisher Scientific), 200 U/mL penicillin, 200 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 µg/mL gentamycin (Sigma-Aldrich), 2.5 µg/mL amphotericin B (Sigma-Aldrich), and mixed with Matrigel® Basement Membrane Matrix (final concentration of Matrigel at 50%; Corning Inc., Corning, NY, USA). Cell suspension was solidified on pre-warmed 24-well culture plates at 37 °C for 30 min. After incubation, medium and supplements were added to each well, and plates were incubated at 37 °C, 5% CO₂. The medium was changed every 2–3 days. TDOs developed within 1–4 weeks and were harvested using Cell Recovery Solution (Corning Inc., Corning, NY, USA), according to the manufacturer's instructions. Pellets were used for RNA extraction.

RNA isolation, sequencing, and data analysis

For RNA sequencing (RNA-Seq), total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendations. RNA purity and quantity were measured by Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham,

MA, USA), and its integrity was evaluated using 4200 TapeStation RNA screens (RNA ScreenTape; Agilent, Santa Clara, CA, USA).

The cDNA libraries were prepared using 100 ng total RNA with the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. IDT for Illumina RNA UD Indexes Set A was added to multiplex the samples, and the paired-end sequencing was performed on Illumina NovaSeq 6000 System (2 x 150 bp). RNA-Seq data quality control was conducted using MultiQC (Ewels et al., 2016). The reads were aligned to the reference genome assembly GRCh38 (Genome Reference Consortium Human Build 38) using the Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al., 2013) and quantified for each Ensembl gene ID using featureCounts (Liao et al., 2014). Only genes with read counts ≥ 1 in at least 90% of samples were retained (23,324 genes out of 60,660). Gene annotation was performed using biomaRt R package (v2.50.3) (Durinck et al., 2009), and Ensembl IDs without corresponding Entrez Gene ID, gene symbol, or gene type were removed. The final count matrix consisted of 23,275 Ensembl IDs (genes). Read count normalization and transformation were performed with the DESeq2 R package (v1.34.0) (Love et al., 2014). Differential gene expression analysis between TDOs and normal ovarian tissue samples was conducted with the limma R package (v3.50.3) (Ritchie et al., 2015). Significantly differentially expressed genes (DEGs) were considered with p -value adjusted to control false discovery rate (FDR) and fold-change (FC) criteria (FDR <0.05 and $|FC| \geq 2$).

For two patients sampled more than once (T7 and T15), the normalized expression levels of annotated genes were compared between the TDOs derived from the same patient based on variance. We selected genes with variance ≥ 5 across samples to identify the most variable during SOC progression.

214

215 **Assessment of external RNA-Seq data**

216 Raw count data of SOC patients (N = 337) was retrieved from The Cancer Genome
 217 Atlas - Ovarian Cancer project (TCGA-OV) using the TCGAbiolinks Bioconductor/R package
 218 (v2.25.2) (Colaprico et al., 2016; Mounir et al., 2019). Clinical data of these patients were
 219 downloaded from Xena Browser (<http://xena.ucsc.edu/>) (Goldman et al., 2020). Raw count data
 220 of healthy ovarian tissue samples were obtained from the Genotype-Tissue Expression (GTEx)
 221 Portal (GTEx Analysis Release V8) (N = 180). These datasets (tumor and normal) were
 222 integrated into a single matrix and analyzed following the same protocol used in our internal
 223 RNA-Seq data. The Ensembl IDs (genes) shared by the TCGA and GTEx data were filtered to
 224 keep only those with read count ≥ 1 in at least 90% of the samples and then annotated with the
 225 biomaRt package (v2.50.3) (Durinck et al., 2009). The final count matrix consisted of 21,834
 226 Ensembl IDs (genes). Next, data normalization and transformation were performed according
 227 to the DESeq2 package protocol (v1.34.0) (Love et al., 2014). Differences in global gene
 228 expression between normal and tumor samples were analyzed using the limma package
 229 (v3.50.3) (Ritchie et al., 2015).

230 Previously published RNA-Seq data from TDOs (culture day 6) derived from malignant
 231 effusions from four patients with HGSOC were obtained upon request (H. Chen et al., 2020).
 232 This dataset was integrated with ours and processed as described earlier. The batch effect
 233 between the two datasets was removed using the ComBat_seq function of sva package (v3.42.0)
 234 (Leek et al., 2012). Global gene expression differences between TDOs (internal, N = 15, and
 235 external TDOs, N = 4) and normal samples (N = 7) were analyzed.

236

237 **Identification of epigenetic regulators**

The differentially expressed lncRNAs and protein-coding genes (PCGs) identified in our TDO samples were filtered for epigenetic regulators according to the EpiFactors database (v2.0; <https://epifactors.autosome.org/>) (Marakulina et al., 2023). Epigenetic regulators included lncRNAs involved in recruiting or sequestering chromatin remodelers, histone modifiers, DNA modifiers, transcription factors, and splicing factors, modulating protein functions, chromatin looping, promoter switching, transcription machinery interference, and RNA or protein binding. We also included PCGs involved in chromatin remodeling, histone and DNA modification, and protein scaffold formation. The DEGs associated with epigenetic regulation were selected for interaction analysis using the ncRNA Functional Annotation Server (ncFANs) database (v2.0; <http://ncfans.gene.ac/>) (Y. Zhang et al., 2021) considering lncRNA interactions with transcription factors and predicted interaction with RNA binding proteins (lncPro score = 80). lncPro scores the lncRNA–protein interaction pairs according to information about nucleotide and amino acid sequence (Lu et al., 2013). The interactions were imported into Cytoscape software (v3.8.2) for visualization, and the network was analyzed using the CytoNCA plugin (v 2.1.6) (Tang et al., 2015), which calculated the nodes' betweenness centrality. Betweenness values range between 0 and 1 and reveal how much each node influences the interactions of other nodes in the network (Doncheva et al., 2012).

OVCAR8 and Ovc316 cell lines

We conducted drug testing using two OC cell lines inherently resistant to carboplatin, namely OVCAR8 and Ovc316. These cell lines were donated by Wan Lam (University of British Columbia, Vancouver, Canada) and Robert Strauss (Danish Cancer Society, Copenhagen, Denmark), respectively. The cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal

bovine serum (Biological Industries, Beit HaEmek, Israel) and 1% antibiotic-antimycotic (10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 ug/mL of Amphotericin B 100x) (Thermo Fisher Scientific, Waltham, MA, USA), at 37°C with 5% CO₂. A PCR-based assay was used to investigate mycoplasma contamination. Cell counting was performed using the Countess II Automated Cell Counter (Thermo Fisher Scientific) based on Trypan blue exclusion.

Dose-response curves and IC₅₀ determination

The epi-drugs decitabine (DAC) (European Medicines Agency, Amsterdam, The Netherlands) and tazemetostat (TAZ) (TargetMol Chemicals, Boston, MA, USA) were diluted in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA). The 50% maximum growth inhibition (IC₅₀) was calculated using GraphPad Prism 7.0 (GraphPad Software, Boston, MA, USA). Approximately 5000 cells/well were seeded with supplemented media at the 96-well optical-bottom plate (Thermo Fisher Scientific, Waltham, MA, USA). After cell adhesion and starvation, DAC and TAZ were added, isolated, or combined at different concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µM). Cell viability was assessed after 24, 48, and 72 hours of treatment using the CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA). The fluorescence values were recorded at 530/590 nm using the Synergy HT microplate reader (BioTek, Winooski, VT, USA). The experiments performed at each time point were repeated in triplicates using DMSO as a control, ensuring the thoroughness of the results.

Cell lines and TDOs' drug testing

Cells (~3 X 10⁵) of both cell lines were exposed to DAC (1µM) and TAZ (2.5µM), isolated or combined, and to control (DMSO) for 96 hours, followed by 24 hours of recovery.

Next, cells were treated with the Gibco trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA), and 5000 cells/well were seeded on the 96-well optical-bottom microplate. Carboplatin (Carb) was added at 50 μ M and 100 μ M for 72h of exposure, followed by cell viability assessment using the CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA) (Supplementary Figure 1A). The Carb dosage was determined according to a previous study of our group that included OVCAR8 and Ovc316 cell lines (Abildgaard et al., 2022).

A similar strategy to the one described for cell lines was applied to TDOs derived from one patient (OV20231201PE) (Supplementary Figure 1B). After TDO generation, large organoids were dissociated using the Gibco TrypLE Express Enzyme (1x), phenol red (Thermo Fisher Scientific, Waltham, MA, USA), and filtered through a 40 μ m cell strainer. Approximately 20,000 cells/well were seeded in 24-well Nunclon Sphera microplates (Thermo Fisher Scientific, Waltham, MA, USA). After 24h of recovery, TDOs were treated with DAC and TAZ, isolated or combined, at 0.5 and 2.5 μ M, respectively, for 72h. After recovering for 24h, TDOs were exposed to Carb at 100 μ M for 72h. Cell viability was assessed, as described above. Luminescence was recorded using the Synergy HT microplate reader (BioTek, Winooski, VT, USA). Drug testing was performed in triplicates with at least two independent experiments.

RNA extraction and RT-qPCR

Total RNA was isolated from TDOs of patients sampled multiple times (T7 and T15) and from OVCAR8 and OVC316 cell lines after DAC and/or TAZ treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA). Expression analyses (RT-qPCR) were performed for *MEG3* (Hs00292028_m1) and *GUSB* (Hs00939627_m1) (Thermo

Fisher Scientific, Waltham, MA, USA) in duplicates on QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), using 10 ng of cDNA and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Relative gene expression values were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Statistical analysis was carried out using the ANOVA t-test; a two-tailed test p -value < 0.05 was considered significant.

Statistical analysis and data visualization

Statistical analyses were performed using the R software (v.4.1.0). Hierarchical clustering analyses based on Euclidean distance were conducted, and heatmaps were generated using the R packages ComplexHeatmap (v.2.10.0)(Gu et al., 2016), cluster (v.2.1.3), and RColorBrewer (v.1.1-3). Gene set enrichment analysis (GSEA) was performed with clusterProfiler R package (v 4.8.3), and the ridge plots were generated using enrichplot R package (v 1.20.1). GSEA was run on the list of the genes obtained from the differential expression analyses pre-ranked based on $-\log_{10}(p\text{-value}) * \text{sign}(\log FC)$. Pathways and ontologies enrichment was established using the DEGs from the internal dataset if the Benjamini–Hochberg adjusted p -value for the GSEA was < 0.05 . The remaining graphs were generated using the ggpubr (v.0.4.0) and ggplot2 (v.3.3.6) R packages. Statistical analyses of the drug testing results were conducted in GraphPad Prism 10 (GraphPad Software, Boston, MA, USA), using ANOVA or Kruskal-Wallis tests (p -value < 0.05).

RESULTS

Ex vivo model establishment and characterization

We successfully generated 18 TDOs from 15 SOC patients (11 HGSOV and 4 LGSOV). Although TDOs were obtained from six other cases, they showed a low proliferation rate and were excluded. Overall, we achieved a success rate of 71.4% in TDO generation. Our patient cohort included individuals diagnosed at different disease stages who underwent diverse therapeutic regimens, including two patients (T4 and T5) from whom we derived TDOs before treatment. Among the SOC patients, 13 were diagnosed with FIGO stages III and IV. Two patients were sampled more than once: patient T7 (HGSOV) had two ascites collected with 45 days of interval; patient T15 (LGSOV) was subjected to ascites drainage three times, with 348 and 65 days of interval. The SOC-derived organoids presented a wide range of morphologies, varying from cystic to dense, with different degrees of circularity and cellular cohesiveness (Fig. 1). None of these histological patterns were associated with patients' clinical characteristics or treatment responses.

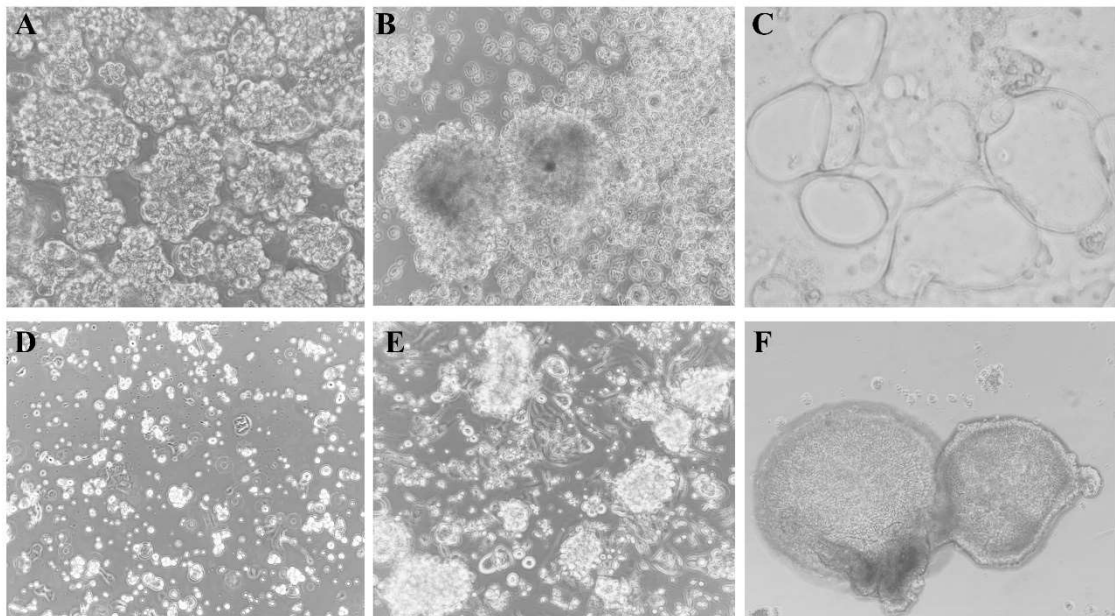


Figure 1. Tumor-derived organoids (TDOs) generated from malignant effusions from serous ovarian carcinoma patients. (A-F) Examples of TDOs exhibiting different

morphologies and degrees of cohesion. (A) and (D) show TDOs on the first day of 3D culture (magnification 40x and 10x, respectively).

RNA-Seq data reveals lncRNAs with decreasing expression levels during SOC course

The comparison between gene expression profiles of TDOs and normal ovarian tissue samples of the internal dataset revealed 7,668 DEGs: 3,586 upregulated and 4,082 downregulated genes in TDOs. A total of 1,428 DEGs corresponded to genes encoding lncRNAs (Table S1), 69% (N=983) with reduced expression levels, while 5,241 were identified as PCGs, in which 55% (N=2,881) showed increased expression levels. An unsupervised clustering analysis based on the profile of differentially expressed lncRNAs revealed two clusters that accurately separated TDOs and normal samples (Fig. 2A).

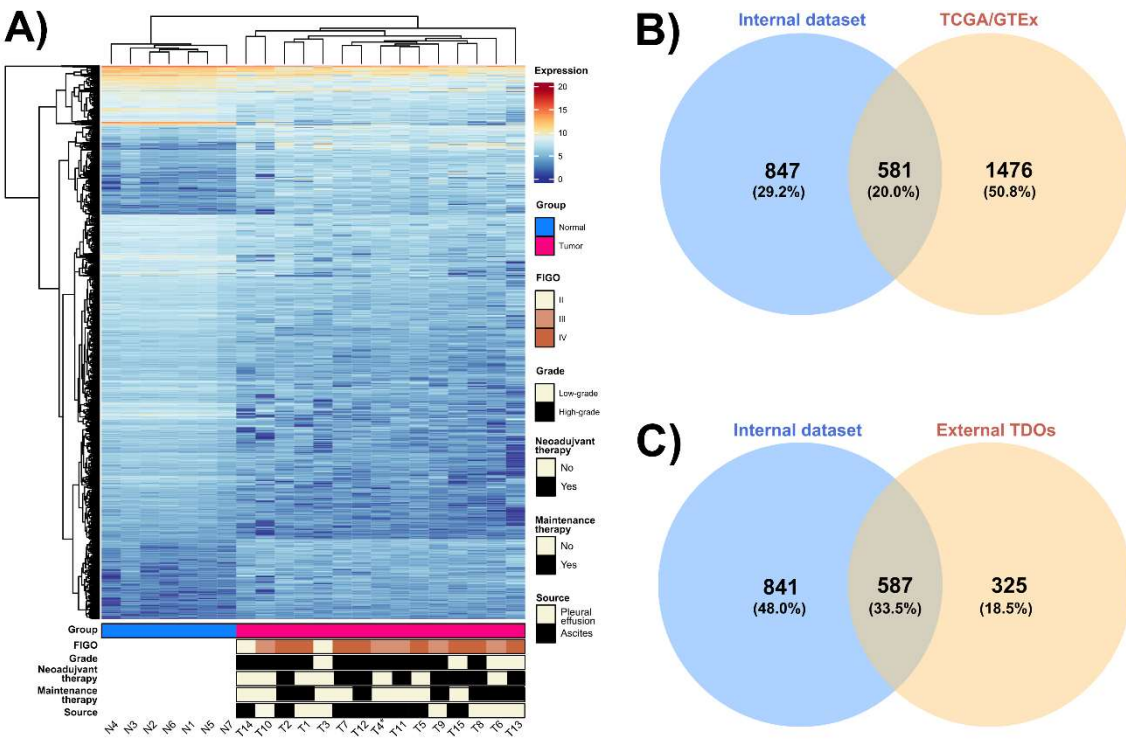


Figure 2. Expression profile of the differentially expressed long noncoding RNAs (lncRNAs) identified in serous ovarian carcinoma. (A) The heatmap depicts lncRNAs differentially expressed in tumor-derived organoids (TDOs) compared with normal ovarian tissue samples from our cohort. Hierarchical clustering was performed assuming Euclidean

distance. Columns and rows represent samples and genes, respectively. Red and blue indicate increased and decreased expression, respectively. The bottom bars show additional variables for each sample: group (normal or tumor), International Federation of Gynecology and Obstetrics (FIGO) stage (II, III or IV), histological grade (high- or low-grade), treatment information (neoadjuvant and/or maintenance therapy), and sample source (ascites or pleural effusion). *T4 was diagnosed with high-grade serous ovarian carcinoma with sarcoma component (carcinosarcoma). The Venn diagrams show the overlap of differentially expressed lncRNAs between the internal dataset and (B) the TCGA/GTEX samples or (C) the external TDOs (H. Chen et al., 2020).

For two patients, one with HGSOC (case T7) and one with LGSOC (case T15), we derived organoids at different time intervals and used variance to determine the most variable genes across TDOs from each patient. The HGSOC patient presented 82 genes while the LGSOC patient presented 113 genes (variance ≥ 5) (Tables S2 and S3). In both SOC cases, *MEG3* (maternally expressed 3) was the lncRNA exhibiting the highest variance throughout the disease course, and its expression levels decreased over time, which was confirmed by RT-qPCR (Supplementary Figure 2). The differential expression analysis of the internal dataset revealed that *MEG3* levels were reduced in TDOs compared to normal tissue samples (Fig. 3A).

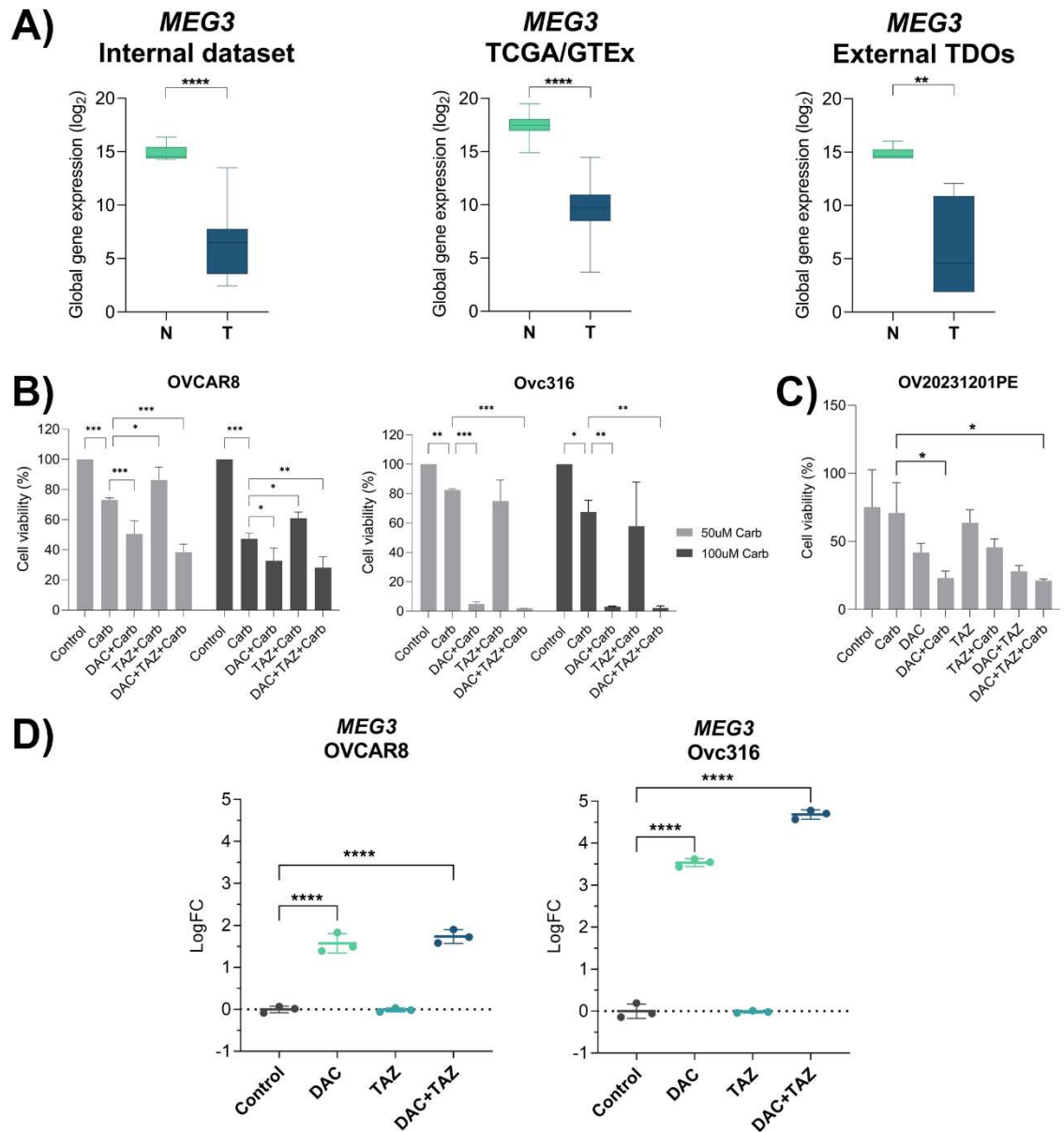


Figure 3. *MEG3* expression in serous ovarian carcinoma (SOC) and effects of epigenetic drugs on carboplatin sensitivity. A) The boxplots depict, from left to right, the expression levels of the long noncoding RNA *MEG3*, analyzed by RNA-Seq, in the internal cohort, TCGA/GTEx samples, and external TDOs compared with our normal ovarian samples. The statistical difference was determined with the Mann–Whitney test. (B) The bar plots represent the viability in ovarian cancer cell lines OVCAR8 and Ovc316 and (C) SOC-derived organoid pre-treated with decitabine (DAC) and tazemetostat (TAZ), isolated or combined and subsequently challenged with carboplatin (Carb). The change in viability was calculated relative to the

treatment with Carb alone. Statistical significance was assessed by ordinary one-way ANOVA with Dunnett's multiple comparisons test (cell lines) or Kruskal-Wallis with Dunn's multiple comparisons test (TDO). D) The dot plots show the *MEG3* expression levels, evaluated by RT-qPCR, in cell lines following the treatment with DAC and TAZ, alone or in combination. The replicates' gene expression fold-change (logFC) was presented as the means $\pm$ standard deviations, represented by the center line and the error bars, respectively. Statistical significance was assessed by one-way ANOVA for all conditions compared to control (DMSO). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

External data supports lncRNA downexpression in SOC and suggests interplay with the epigenetic machinery

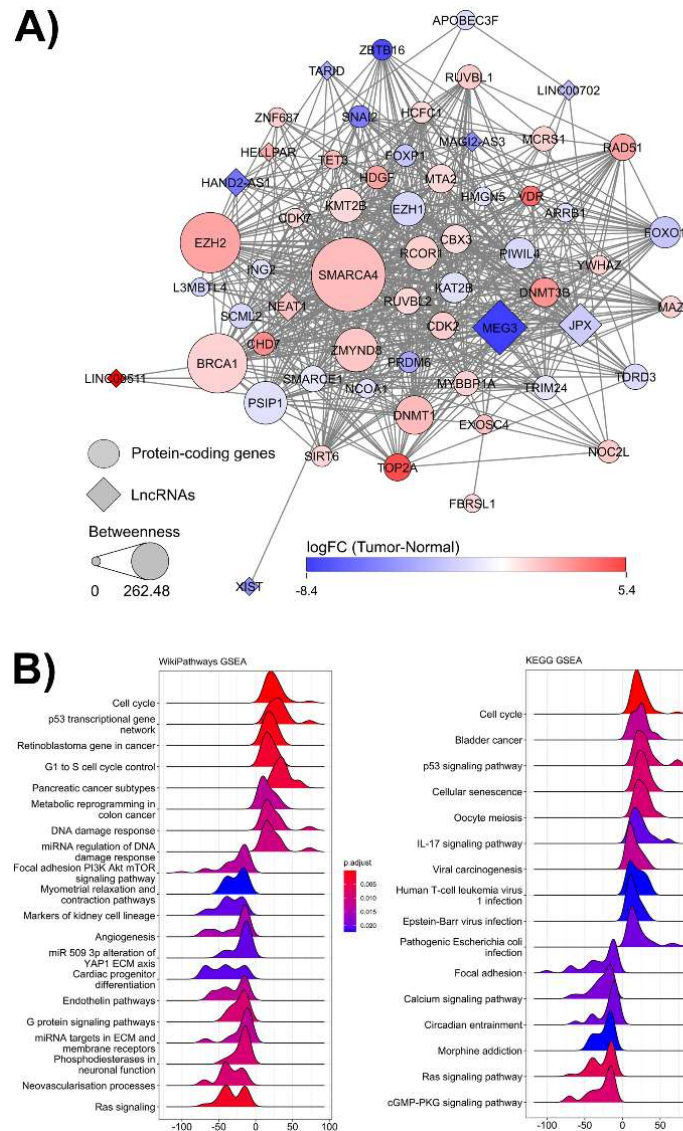
To cross-validate our findings from SOC-derived organoids, we analyzed external RNA-Seq data from public repositories and SOC-derived organoids generated in another study (H. Chen et al., 2020). The differential expression analysis comparing SOC samples from TCGA-OV (N = 337) with healthy ovarian tissue samples from GTEx (N = 180) detected 11,143 DEGs, of which 5,812 showed increased expression and 5,331 exhibited decreased expression in tumors. Among the DEGs, 2,057 were lncRNA genes (Supplementary Figure 3A), and 7,460 were PCGs. A comparison with the internal dataset resulted in 581 lncRNAs (20%) differentially expressed in both cohorts (Fig. 2B). Most of these lncRNA genes (N = 373) showed decreased expression, while 148 exhibited augmented expression in SOC samples and TDOs. In parallel, 3,490 PCGs (37.9%) were differentially expressed in both cohorts, of which 3,404 showed concordant expression profiles (1,846 increased and 1,558 decreased expression levels in SOC samples and TDOs).

The differential expression analysis using the TDOs dataset from Chen *et al.* (2020) and our normal samples revealed 4,653 DEGs: 1,727 were overexpressed, and 2,926 were

downexpressed in TDOs. In this set of genes, 912 encoded lncRNAs (Supplementary Figure 3B) and 3,262 encoded proteins. Moreover, 587 lncRNA genes (33.5%) (Fig. 2C) and 3,131 PCGs (58.3%) were differentially expressed in both studies. Similarly to the TCGA/GTEX dataset, most DEGs encoding lncRNAs exhibited reduced levels (N = 450), while 134 showed increased levels in the external and internal TDOs. Considering the differentially expressed PCGs, 1,604 were downexpressed, and 1,527 were overexpressed in TDOs from both studies. Consistent with the results from our TDOs, lncRNA *MEG3* showed reduced expression in TCGA SOC tissue samples and external TDOs (Fig. 3A).

We obtained a list of epigenetic regulators from the EpiFactors database to compare with the set of DEGs detected in our internal dataset. The lists of differentially expressed lncRNAs and PCGs associated with epigenetic regulation were included in Tables S4 and S5, respectively. According to EpiFactors, seven down expressed lncRNAs (*MEG3*, *JPX*, *TARID*, *XIST*, *LINC00702*, *MAGI2-AS3*, and *HAND2-AS1*) and five overexpressed lncRNAs (*LINC00511*, *GNAS-AS1*, *LASTR*, *NEAT1*, and *HELLPAR*) were involved in the epigenetic regulation. Moreover, 68 PCGs with reduced expression and 100 PCGs with increased expression were involved in epigenetic processes. The overexpressed PCGs included several acknowledged components of the epigenetic machinery, such as DNA methyltransferases (*DNMT1* and *DNMT3B*), histone methyltransferase *EZH2* (enhancer of zeste 2 polycomb repressive complex 2 subunit), histone deacetylases (*HDAC7* and *HDAC9*), and histone demethylases (*KDM1B* and *KDM2A*). The enhanced expression of *DNMT3B*, *EZH2*, and *HDAC9* was also observed in SOC samples from TCGA and external TDOs (Supplementary figure 4). Next, we used the lncPro method, embedded in the ncFANs database to predict interactions between lncRNAs and proteins implicated in epigenetic regulation and constructed a network based on these results. *MEG3* was the lncRNA with the highest centrality in the generated network, and it was predicted to directly interact with 20 different epigenetic

effectors, including EZH2 and DMT3B (Fig. 4A). These findings support the *MEG3* function as a hub of epigenetic regulation; therefore, its reduced levels in SOC potentially interfere with the modulation of downstream target genes, including those involved in cancer progression and therapy response.



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Figure 4. Prediction of interactions between epigenetic regulators and identification of altered pathways in tumor-derived organoids (TDOs). A) Interaction network of long noncoding RNAs and protein-coding genes differentially expressed in TDOs and associated with epigenetic regulation. Larger circles indicate a higher betweenness centrality value of the node. Red and blue intensities represent gene expression fold-change (logFC). Gray lines

447

highlight the interactions predicted using the ncRNA Functional Annotation Server (ncFANs) database. B) The ridge plots show the pathways most significantly enriched for the differentially expressed genes detected in our TDOs based on gene set enrichment analysis (GSEA).

The list of DEGs detected in our TDOs underwent gene set enrichment analysis, which resulted in 37 terms from the WikiPathways and 16 terms from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database that were significantly enriched (Fig. 4B). The top pathways associated with overexpressed genes in TDOs included cell cycle, p53 signaling, bladder cancer, and *RB* gene in cancer, while the pathways related to downexpressed genes included Ras signaling, neovascularization processes, and cGMP-PKG signaling.

Epigenetic priming modifies the resistance to carboplatin in OC cell lines and SOC-derived organoids

Based on the epigenetic effectors found to be overexpressed in our TDOs, we investigated if inhibiting a couple of these components of the epigenetic machinery could modulate the response to chemotherapeutic drugs in OC cell lines and SOC-derived TDOs by promoting the re-expression of epigenetically silenced genes. To this end, we selected doses of epi-drugs that maximized the epigenetic re-programming without producing cytotoxic effects since we intended to perform a pretreatment followed by the challenge with Carb. After exposing OC cells to DNA methyltransferase inhibitor DAC and EZH2 inhibitor TAZ, isolated or in combination, for 96h, we determined that OVCAR8 and Ovc316 IC₅₀ values were 6.006 uM and 1.545 uM for DAC, 12.17 uM and 7.42 uM for TAZ, and 5.776 uM and 6.411 uM for the combined epi-drugs, respectively. We selected DAC 1 µM and TAZ 2.5 µM, respectively, as the higher doses that did not affect cell viability (Supplementary Figure 5 A-B). Based on a previous study of our group (Abildgaard et al., 2022), we exposed the cell lines OVCAR8 and Ovc316 to Carb at 50 and 100 µM.

We also investigated the effects of epigenetic priming with DAC and TAZ on the viability of OVCAR8 and Ovc316 cell lines. The priming with DAC administered alone or in combination with TAZ promoted the re-sensitization to Carb at 50 and 100 μ M in both cell lines (Fig. 3B). The TDO OV20231201PE exhibited increased sensitivity to Carb after pretreatment with DAC alone or in combination with TAZ (Fig.3C).

lncRNA *MEG3* expression increased after epigenetic priming

The expression of the lncRNA *MEG3* was investigated to evaluate whether the epigenetic priming affected its expression. Both OVCAR8 and Ovc316 cell lines showed significantly higher *MEG3* expression after treatment with DAC isolated or combined with TAZ when compared to the control (DMSO) (Fig. 3D).

DISCUSSION

Tumor-derived organoid models have drawn increasing attention in basic and translational cancer research since they are expected to reflect the inter- and intra-patient heterogeneity of human cancers, resembling the lesion of origin from the histopathological, genetic and phenotypic perspectives (Drost & Clevers, 2018; LeSavage et al., 2022). Moreover, TDOs allow experimental manipulability while they recapitulate the biological complexity, filling a gap between conventional two-dimensional cell culture and animal models (M. Li & Izpisua Belmonte, 2019). To date, several studies have generated OC organoids attaining an overall success rate of 30-90% and a success rate of 58.3% to nearly 100% for organoids derived exclusively from ascites or pleural effusion (Bi et al., 2021; Hill et al., 2018; Qin et al., 2022; Senkowski et al., 2023). The variability in success rates is not unexpected, given the current

lack of standardized medium formulations and procedures for OC organoid culture establishment and maintenance (Qin et al., 2022).

We established TDOs from malignant effusions for 15 out of 21 SOC patients, achieving a 71.4% success rate, and expanded the cohort compared to other studies, which included up to 12 OC patients for organoid derivation using malignant fluids (Bi et al., 2021; H. Chen et al., 2020; de Witte et al., 2020; Hill et al., 2018; Senkowski et al., 2023). Also, the morphology of our TDOs markedly differed between our patients, as expected for SOC-derived organoids (Tao et al., 2022). As a result, we established a TDO repertoire that captured the SOC heterogeneity and investigated abnormally expressed genes, especially lncRNAs, which are key for expression modulation and consequently impact several biological processes.

Although there are ubiquitously expressed lncRNAs, these transcripts often exhibit higher spatial and temporal specificity than mRNAs, which is consistent with their role in determining cell state and differentiation program under physiological and pathophysiological conditions, such as cancer (Mattick et al., 2023). These features reinforce the potential of lncRNAs as biomarkers and therapeutic targets in diverse diseases, including OC, whose outcomes have been associated with lncRNAs individually or as part of molecular signatures (Calanca et al., 2020; Malgundkar & Tamimi, 2024). Herein, 1,428 lncRNA were differentially expressed, mostly downexpressed in TDOs. To cross-validate the differential expression analysis results, we compared our findings with a set of SOC and normal ovarian samples from TCGA/GTEX and four TDOs generated by Chen *et al.* (2020) (H. Chen et al., 2020). Among the 983 lncRNAs exhibiting reduced expression in our TDOs, 373 and 450 were downexpressed in SOC samples from TCGA and external TDOs, respectively. LncRNA expression levels were also evaluated throughout the clinical course for two patients of our cohort, and we verified that *MEG3* was the lncRNA with the highest variance across TDOs in both cases. *MEG3* expression decreased over time, while both patients presented progressive disease and died from SOC. The

external data confirmed *MEG3* down-expression in SOC samples and TDOs. Accordingly, Sheng et al. (2014) showed that *MEG3* expression was lost in 70% of OC tissue samples, primarily due to abnormal promoter hypermethylation (Sheng et al., 2014). Lower levels of *MEG3* were correlated with advanced FIGO stages and reduced overall survival in OC patients (L. Wang et al., 2019; Xiu et al., 2017). Altogether, this evidence suggests that reduced *MEG3* expression is associated with SOC progression and that epigenetic silencing is likely responsible for this lncRNA downregulation.

The expression of the imprinted gene *MEG3* was usually lost or reduced in a wide variety of human cancers (Ghafouri-Fard & Taheri, 2019; Zhou et al., 2012). The *locus* encoding this lncRNA contains several maternally and paternally expressed genes and is controlled by two differentially methylated regions (DMRs): the intergenic DMR, upstream from the *MEG3* transcription start site, and the *MEG3*-DMR, which overlaps with *MEG3* promoter (Al-Rugeebah et al., 2019; Zhou et al., 2012). *MEG3* was acknowledged as a tumor suppressor due to its pro-apoptotic and anti-proliferative activities *in vitro* and *in vivo* (Ghafouri-Fard & Taheri, 2019; Zhou et al., 2012). To exert these functions, nuclear retained lncRNAs, such as *MEG3*, can recruit or sequester proteins that affect transcription through epigenetic regulatory processes, including DNA methylation, chromatin remodeling, and histone marks deposition (Cabili et al., 2015; Morlando & Fatica, 2018; Statello et al., 2021; Zhou et al., 2020). Among the DEGs identified in our TDOs, we detected 180 previously characterized as epigenetic regulators, encompassing *MEG3* and 11 additional lncRNAs, and 168 PCGs. Experimental evidence showed that *MEG3* binds to EZH2 in prostate, breast, gallbladder, pancreatic, and lung cancer cells with consequences for cell proliferation, migration, and invasion (Iyer et al., 2017; D.-Q. Li et al., 2022; Mondal et al., 2015; Terashima et al., 2017; Zhou et al., 2020). EZH2 is a repressive histone modifier, part of polycomb repressive complex 2 (PRC2), which catalyzes H3K27 methylation promoting gene silencing

and is often upregulated in cancer, especially in advanced stages (Margueron & Reinberg, 2011). Using a computational approach (Lu et al., 2013) to explore the interactions between the epigenetic regulators abnormally expressed in our TDOs, *MEG3* was predicted to interact with 20 protein regulators, including EZH2 and DNA methyltransferase 3 beta (DNMT3B). DNMT3B contributes to establishing *de novo* DNA methylation profiles at previously unmethylated loci, it is crucial during embryonic development and usually presents augmented expression in human cancers (Gagliardi et al., 2018; Gujar et al., 2019). In accordance with prior reports, we observed that genes *EZH2* and *DNMT3B* were overexpressed in SOC, in the internal and external TDOs and SOC tissue samples. Therefore, *MEG3* is likely a hub for fine-tuning gene expression through epigenetic mechanisms, and its downregulation prevents this lncRNA from playing tumor-suppressive roles that could control SOC aggressive behavior.

Previous evidence suggests that the expression levels of *MEG3*, along with *PVT1* and *TUG1*, can predict OC patients' resistance to platinum-based chemotherapy (El-Khazragy et al., 2020). Zhang et al. (2017) detected increased *MEG3* expression and lessened resistance to cisplatin after treating platinum-resistant OC cells with curcumin (J. Zhang et al., 2017). Curcumin served as a demethylating agent, reversing *MEG3* promoter hypermethylation and restoring the lncRNA expression. Herein, OC cell lines characterized as carboplatin-resistant (Abildgaard et al., 2022) were treated with two FDA-approved epi-drugs, one broad and one narrowed spectrum epigenetic re-programmer, DAC and TAZ, respectively. DAC promotes genome-wide changes in gene expression functioning as a cytidine analog that is incorporated into DNA-by-DNA methyltransferases (DNMTs), while TAZ inhibits specifically the enzymatic activity of EZH2 (Jin et al., 2021; C. Li et al., 2021). Subsequently, we assessed the expression of *MEG3* and sensitivity to Carb in the OC cells. OVCAR8 and Ovc316 cell lines showed significantly higher *MEG3* expression after the treatment with DAC alone or in combination with TAZ. Additionally, both cell lines exhibited enhanced sensitivity to Carb

following the pretreatment with DAC, isolated or combined with TAZ, while no significant effect was verified after the administration of TAZ individually. Most approved drugs targeting epigenetic regulators are applied to treat hematologic malignancies since studies on therapeutic potential in solid tumors are limited (Jin et al., 2021). However, recent clinical trials showed that selected groups of platinum-resistant OC patients benefited from epigenetic priming with DNMT inhibitors (S. Chen et al., 2022; Oza et al., 2020). On the other hand, EZH2 inhibitors alone did not show effective antitumor activity in solid tumors with augmented *EZH2* expression, including ovarian cancer (C. Li et al., 2021). One TDO derived from malignant effusions also received the same treatment regimen administered to the cell lines. We found an increased sensitivity to Carb after pretreatment with DAC alone or in combination with TAZ. To the best of our knowledge, ours is the first study to test this treatment regimen in organoids derived from OC patients. Recently, Gerton et al. (2023) combined DNMT inhibitor 5-azacytidine and HDAC inhibitor givinostat with immune checkpoint blockade to treat spheroids derived from ascites of HGSOc patients (Gerton et al., 2023). Their findings suggested that the use of epi-drugs could potentiate the immunotherapy response. To provide more robust evidence of epi-drugs' efficacy in restoring platinum sensitivity and to capture inter- and intra-patient heterogeneity, it would be ideal to expand the number of TDOs exposed to our treatment approach. Also, to determine whether the increased cytotoxic effects of Carb in OC cells and SOC-derived organoids are due to *MEG3* enhanced levels, a target-specific strategy inducing *MEG3* re-expression would be necessary.

The TDOs we generated are promising models to investigate how the *MEG3* re-expression could undermine SOC resistance to therapy. Buttarelli et al. (2020) showed that inducing *MEG3* expression *in vitro* reverted the malignant phenotype in HGSOc, inhibiting the growth of spheroids derived from the HEY cell line (Buttarelli et al., 2020). Spheroids are simpler and homogeneous 3D models compared to organoids, since they fail to reproduce tissue

architecture and lack the presence of multiple cell types (Porter et al., 2020). Thus, TDOs are a better alternative to evaluate *MEG3* re-expression considering SOC inherent heterogeneity. Besides the application in the preclinical setting, the SOC-derived organoids could be used in clinical trials if the results of targeted *MEG3* modulation were favorable. Although incipient, the use of TDOs to select individualized cancer treatment seems promising and has been reported by two prospective clinical trials with colorectal cancer patients (Jensen et al., 2023; Ooft et al., 2021), while several ongoing trials are set out to establish organoid-guided protocols to treat diverse tumor types, including pancreatic, ovarian, gastric, and breast cancer (ClinicalTrials.gov IDs: NCT04931394, NCT05813509, NCT05842187, NCT06102824). Recently, the first case employing ascites-derived organoids to guide the personalized treatment of a patient with recurrent HGSOC was reported (W. Chen et al., 2023). The TDO was used for drug sensitivity screening, and following the treatment, the levels of serum biomarker CA125 normalized while the patient's condition improved according to imaging exams. Therefore, TDOs can help identify new clinically actionable targets and test therapeutic approaches for HGSOC in a patient-specific manner, assisting in translating basic research findings into patient treatment.

CONCLUSIONS

In summary, we have successfully derived organoids from malignant effusions of SOC patients with a high success rate. These innovative models maintain the intra- and inter-patient heterogeneity, allowing a trustworthy characterization of the SOC transcriptome. Our analyses uncovered a set of altered epigenetic regulators, including lncRNAs and PCGs. We focused on the interplay between these regulators since they are responsible for fine-tuning cellular transcriptional programs, interfering with processes that determine SOC behavior. In particular,

the lncRNA *MEG3*, which is downexpressed in TDOs, is potentially involved in epigenetic regulation and therapy response in SOC. Our findings prompt further exploration to determine whether re-expression of *MEG3* could counteract SOC chemoresistance and if this lncRNA could be targeted for clinical intervention.

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959 **DECLARATIONS**

960 **Ethics approval and consent to participate**

961 All patients provided written informed consent, and the trial was conducted following the
962 guidelines for Good Clinical Practice and the Declaration of Helsinki. The Ethics Committee
963 of the Region of Southern Denmark (S-20190102) and The Danish Data Protection Agency
964 (Case No. 19/37168) approved the study.

965

966 **Competing interests**

967 None to report.

968

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974

975 **Availability of data and materials**

976 The original contributions presented in the study are included in the article/supplementary
977 material, further inquiries can be directed to the corresponding author. The datasets generated
978 and/or analyzed during the current study are not publicly available since the publication of
979 individual sequencing data is not approved by the Danish Data Protection Agency.

980

981 **Competing interests**

982 The authors declare that they have no competing interests.

983

984 **Authors' contributions**

985 Conception (S.R.R.), design (S.R.R, M.W., K.D.S., L.H.J., C.A.R.), acquisition (N.C., D.K.M.,
986 R.A.R.V.), analysis (N.C., D.K.M., D.B., M.M.A, M.W.), data interpretation (all), draft (N.C.,
987 S.R.R.), supervision (S.R.R., C.A.R.), project administration and funding acquisition (S.R.R.).
988 All authors read and approved the final version of the manuscript.

989

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Table 1. Clinical data and treatment information for 15 serous ovarian cancer patients included in the study.

Case	Age at diagnosis (years)	Source	FIGO Stage	Neoadjuvant first line chemotherapy	Maintenance therapy after first line	Treatment (cycles)	Last treatment
HGSOC							
T1	69	Pleural effusion	IV	No - operated at diagnosis primary	Yes	Taxol/Carboplatin + Bevacizumab from sixth cycle (6)	Carboplatin
T2	52	Ascites	IV	Yes	Yes (Bevacizumab)	Taxol/Carboplatin (+ Bevacizumab) (6)	
T4	64	Ascites	IIIC	No - operated at diagnosis primary	No	Taxol/Carboplatin (1)	Carboplatin/taxol
T5	71	Ascites	IV	No	No	Untreated	No treatment
T7 [#]	65	Ascites	IV	Yes	No	Carboplatin + Paclitaxel (8)	-
T8	37	Pleural effusion	IV	Yes	Yes (Bevacizumab)	Neoadjuvant Taxol/Carboplatin (6) Bevacizumab maintenance (4) Liposomal doxorubicin (6) Carboplatin (4) Topotecan (3)	Topotecan
T9	69	Pleural effusion	IIIB	Yes - Change to pure palliative after the first 3 cycles	Yes (Bevacizumab)	Taxol/Carboplatin (6) Bevacizumab maintenance (6) Treosulfan (2)	Treosulfan
T10	75	Pleural effusion	IIIC	No - operated at diagnosis primary	No	Taxol/Carboplatin (6) Liposomal doxorubicin/ carboplatin (5)	Carboplatin
T11	76	Ascites	IIIC	Yes - Changes to pure palliative after the first 3 cycles	No	Taxol/Carboplatin (5)	Carboplatin
T12	70	Ascites	IV	Yes	Yes (Bevacizumab followed by Olaparib)	Taxol/Carboplatin (6) Olaparib (6) Caelyx (2) Topotecan (6)	Treosulfan

T14	83	Ascites	IIB	No - operated at diagnosis primary	No	Treosulfan (2) Carboplatin (6)	Carboplatin
LGSOC							
T3	60	Pleural effusion	IIC	No - operated at diagnosis primary	No	Taxol/Carboplatin (6) Carboplatin (6) Carboplatin (6) Carboplatin (6) Bevacizumab monotherapy (6)	Carboplatin
T6	53	Pleural effusion	IIIC	No - operated at diagnosis primary	Yes (Bevacizumab)	Bevacizumab + tocotrienol (9)	Topotecan
T13	76	Pleural effusion	IV	Yes	Yes (Bevacizumab)	Carboplatin (3)	Bevacizumab
T15 [#]	58	Ascites	IV	Yes - Changes to pure palliative after the first 3 cycles	No	Taxol/Carboplatin (8) Liposomal doxorubicin (4) Topotecan (4) Paclitaxel weekly (28) Mekinist Carboplatin (2) Letrozol (oral daily)	Letrozol

FIGO: International Federation of Gynecology and Obstetrics; HGSOC: high-grade serous ovarian carcinoma; LGSOC: low-grade serous ovarian carcinoma; [#] Patients sampled more than once.

7. Perspectivas

Nos últimos anos, novos bancos de dados dedicados a lncRNAs foram desenvolvidos, enquanto outros foram continuamente atualizados, expandindo suas funcionalidades e sua cobertura em termos de quantidade de transcritos. Dessa forma, uma nova versão do guia de bancos de dados compilado no capítulo 1 poderia ser útil para incorporar outras formas de explorar conjuntos dados relacionados a lncRNAs de forma amigável.

Quanto ao capítulo 2, resultados experimentais e evidências recuperadas de bancos de dados sugeriram que *SNHG12* pode estar interagindo com HDAC9, uma proteína da maquinaria epigenética. Essa interação promoveria a remoção de modificações pós-traducionais em histonas e interferiria na expressão de genes que ocupam regiões próximas ao *locus* desse lncRNA. Contudo, até o momento, nenhum estudo funcional abordou a interação entre *SNHG12* e HDAC9. A validação da interação com HDAC9 poderia explicar a participação de *SNHG12* na resistência à terapia baseada em platina no OC, considerando que nossos resultados mostraram que o silenciamento desse lncRNA melhora a resposta à carboplatina nas linhagens celulares de OC.

Para o avanço do conhecimento obtido com o nosso estudo descrito no capítulo 3, seria relevante demonstrar o papel causal de *MEG3* na quimiorresistência. Para isso, poderia ser utilizada uma abordagem alvo-específica que induziria a reexpressão desse lncRNA. Assim, seria possível comprovar se a modulação da resposta à carboplatina está diretamente relacionada com seu nível de expressão no OC. Uma vez que as drogas testadas nesse estudo interferem não-seletivamente com a expressão de genes regulados por mecanismos epigenéticos, o uso da edição epigenética por meio do sistema CRISPR/Cas (do inglês: *Clustered Regulatory Interspaced Short Palindromic Repeats/ CRISPR-associated*), por exemplo, seria uma alternativa para reprogramar especificamente o lncRNA de interesse ⁸⁹.

Conforme discutimos em um artigo prévio do nosso grupo, os efeitos transitórios e globais das *epi-drugs* podem ser contrabalanceados por abordagens que modulem de forma *locus*-específica a expressão de lncRNAs candidatos ⁹⁰. Esse tipo de abordagem pode revelar o potencial de *MEG3* como alvo para ressensibilizar as pacientes com SOC ao tratamento convencional e, eventualmente, ser adotada no contexto clínico em combinação com outras estratégias terapêuticas.

8. Conclusões

- A utilização de bancos de dados públicos dedicados a lncRNAs para buscar transcritos desregulados no OC permitiu a identificação de um conjunto de candidatos que interagem com a maquinaria epigenética, como anteriormente sugerido por evidências experimentais e computacionais;
- Com base nos resultados obtidos, verificamos que o lncRNA *SNHG12* contribui para a modulação da resposta à carboplatina nas linhagens celulares de câncer de ovário estudadas;
- Foi possível estabelecer TDOs a partir de amostras de efusões malignas de 15 pacientes com SOC. Nesses modelos experimentais, identificamos a expressão diminuída do lncRNA *MEG3*, que foi confirmada em dados externos derivados de SOC;
- Análises usando bancos de dados permitiram prever a interação de *MEG3* com efetores epigenéticos também diferencialmente expressos nos TDOs. Portanto, *MEG3* está potencialmente implicado na regulação epigenética;
- Após o tratamento de linhagens celulares resistentes à carboplatina com *epi-drugs*, verificamos a resposta ao quimioterápico e o aumento dos níveis de *MEG3*, sugerindo a participação desse lncRNA na resposta ao tratamento dos carcinomas serosos de ovário.

9. Referências

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10. Material Suplementar

Os materiais suplementares referentes a cada capítulo estão disponíveis *online* com acesso pelo link a seguir. Para acessá-los, basta copiar e colar o endereço do link no navegador.

https://reitoriaunespbr-my.sharepoint.com/:f:/g/personal/naiade_calanca_unesp_br/EoVcPPFbX8JCjEMQUMUcbp8BKUvf2u_1PMXoOrP7Jlad1w?e=Eb08MK.

Os materiais suplementares dos artigos já publicados também podem ser acessados *online* no *site* do periódico.

11. Anexo

11.1. Outros artigos publicados:

11.1.1 **Calanca, N.;** Francisco, A.L.N.; Bizinelli, D.; Kuasne, H.; Barros Filho, M.C.; Flores, B.C.T.; Pinto, C.A.L.; Rainho, C.A.; Soares, M.B.P.; Marchi, F.A.; Kowalski, L.P.; Rogatto, S.R. DNA methylation-based depiction of the immune microenvironment and immune-associated long non-coding RNAs in oral cavity squamous cell carcinomas. *Biomed Pharmacother.* 2023 Nov;167:115559. doi: 10.1016/j.biopha.2023.115559. Fator de impacto (JCR 2022): 7,5; Citações (Scopus): 1.

11.1.2 **Calanca, N.;** Binato, S.M.S.; Silva, S.D.; Brentani, H.P.; Sennes, L.U.; Pinto, C.A.L.; Domingues, M.A.C.; Fonseca-Alves, C.E.; Rainho, C.A.; Rogatto, S.R. Master regulators of epithelial-mesenchymal transition and WNT signaling pathways in juvenile nasopharyngeal angiofibromas. *Biomedicines.* 2021, 9, 1258. doi: 10.3390/biomedicines9091258. Fator de impacto (JCR 2022): 4,7; Citações (Scopus): 3.

11.2. Manuscrito submetido:

11.2.1 **Calanca, N.;** Faldoni, F.L.C.; Souza, C.P.; Souza, J.S.; Alves, B.E.S.; Soares, M.B.P.; Wong, D.V.T.; Lima-Junior, R.C.P.; Marchi, F.A.; Rainho, C.A.; Rogatto, S.R. Inflammatory breast cancer microenvironment repertoire based on DNA methylation data deconvolution reveals actionable targets to enhance the treatment efficacy. *J Transl Med.* Fator de impacto (JCR 2022): 7,4 (*under review*)