

PROGRAMA DOUTORAL

CIÊNCIAS BIOMÉDICAS

Unveiling the molecular networks behind gastric cancer stem cells: the path for therapy success

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D
2023



Diana Sofia de Sá Pádua. Unveiling the molecular networks behind gastric cancer stem cells: the path for therapy success



D.ICBAS 2023

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UNVEILING THE MOLECULAR NETWORKS BEHIND GASTRIC CANCER STEM CELLS: THE PATH FOR THERAPY SUCCESS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas;
Programa Doutoral da Universidade do Porto (Instituto de
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Diana Sofia do Sá Pádua

FINANCIAMENTO

O trabalho apresentado nesta tese foi desenvolvido no Instituto de Patologia e Imunologia Molecular da Universidade do Porto (Ipatimup) / Instituto de Investigação e Inovação em Saúde (i3S) da Universidade do Porto. E foi financiado através de uma Bolsa de Doutoramento com a referência SFRH/BD/146186/2019 concedida pela Fundação para a Ciência e a Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Ensino Superior de Portugal e cofinanciada pelo Fundo Social Europeu (FSE) através do Programa Operacional Regional Norte.





AUTHOR DECLARATIONS

PERCEITOS LEGAIS

The author declares to have actively participated in the conception and execution of the work that originated the data presented in this thesis, as well as in its interpretation, discussion and writing.

The author declares that this thesis is in accordance with the terms of the national legislation n. º 2, alínea a) do artigo 31º do Decreto-Lei n. º 115/2013 de 7 de Agosto. The work presented in this thesis is published in peer-reviewed scientific journals and one article is in preparation for publication (as presented in the List of Publications section of this thesis).

Furthermore, the author declares that some data and conclusions of the article titled “A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin” (Chapter II of this thesis), published in 2020 in the peer-reviewed journal *Cancers*, were contained in her master's thesis titled “Isolation and characterization of gastric cancer stem cells based on SOX2/OCT4 activity” from the Master's in Biochemistry (Faculty of Sciences of the University of Porto and ICBAS) defended in 2017.

LIST OF PUBLICATIONS

Original research article – Published

Diana Pádua, Rita Barros, Ana Luísa Amaral, Patrícia Mesquita, Ana Filipa Freire, Mafalda Sousa, André Filipe Maia, Inês Caiado, Hugo Fernandes, António Pombinho, Carlos Filipe Pereira, Raquel Almeida (2020). A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin. *Cancers*, 12(2), 495.

<https://doi.org/10.3390/cancers12020495>

Author Contributions: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing, visualization, project administration.

Original research article – Published

Diana Pádua, Débora Filipa Pinto, Paula Figueira, Carlos Filipe Pereira, Raquel Almeida, Patrícia Mesquita (2021). HMGA1 Has Predictive Value in Response to Chemotherapy in Gastric Cancer. *Current Oncology*, 29(1), 56–67.

<https://doi.org/10.3390/currncol29010005>

Author Contributions: Conceptualization, methodology, validation, formal analysis, investigation, writing—original draft preparation, writing—review and editing, visualization, project administration.

Original research article – Published

Diana Pádua, Paula Figueira, Mariana Pinto, André Filipe Maia, Joana Peixoto, Raquel T. Lima, António Pombinho, Carlos Filipe Pereira, Raquel Almeida, Patrícia Mesquita (2023). High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-like Cells. *Cancers*, 15(17), 4406.

<https://doi.org/10.3390/cancers15174406>

Author Contributions: Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing, visualization, project administration, funding acquisition.

Original research article – In Preparation

Diana Pádua, Paula Figueira, António Pombinho, Inês Monteiro, Carlos Filipe Pereira, Raquel Almeida, Patrícia Mesquita. Upregulation of HMGA1 confers cancer stem-like features to gastric non-CSCs.

Author Contributions: Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing, visualization, project administration, funding acquisition.

Review article – Published

Diana Pádua, Paula Figueira, Inês Ribeiro, Raquel Almeida, Patrícia Mesquita (2020). The Relevance of Transcription Factors in Gastric and Colorectal Cancer Stem Cells Identification and Eradication. *Frontiers in Cell and Developmental Biology*, 8, 442. <https://doi.org/10.3389/fcell.2020.00442>

Author Contributions: Made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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In addition to the scientific works previously mentioned and which are part of this thesis, during the doctoral period, the author of this thesis also contributed to the following research articles in related fields:

Sara Ribeirinho-Soares, **Diana Pádua**, Ana Luísa Amaral, Elvia Valentini, Daniela Azevedo, Cristiana Marques, Rita Barros, Filipa Macedo, Patrícia Mesquita, Raquel Almeida (2021). Prognostic significance of MUC2, CDX2 and SOX2 in stage II colorectal cancer patients. BMC cancer, 21(1), 359. <https://doi.org/10.1186/s12885-021-08070-6>

Taciane Barbosa Henriques, Diandra Zipinotti dos Santos, Isabella dos Santos Guimarães, Nayara Gusmão Tessarollo, Paulo Cilas Moraes Lyra-Junior, Patricia Mesquita, **Diana Pádua**, Ana Luisa Amaral, Bruno Cavadas, Luisa Pereira, Ian Victor Silva, Raquel Maria da Silva Graça Almeida, Leticia Batista Azevedo Rangel (2021). Inhibition of CXCR2 plays a pivotal role in re-sensitizing ovarian cancer to cisplatin treatment. Aging, 13(10), 13405–13420. <https://doi.org/10.18632/aging.203074>

Patrícia Mesquita, Catarina de Oliveira, Luís F. C. Póvoas, Nelson Leça, Diana Ferreira, **Diana Pádua**, Ricardo Coelho, Ana S. Ribeiro, Joana Paredes, Carmen Jerónimo, Carlos Conde, Bruno Pereira, Raquel Almeida. YTHDF3 regulates MISP expression and impacts gastric cancer cell migration – *In Preparation*

“The most difficult thing is the decision to act, the rest is merely tenacity. The fears are paper tigers. You can do anything you decide to do. You can act to change and control your life; and the procedure, the process is its own reward.”

- Amelia Earhart

AGRADECIMENTOS

Chega ao fim mais um excelente desafio no meu percurso académico e profissional, o Doutoramento. Indubitavelmente, um desafio sinusoidal, com altos e baixos, que para além do crescimento intelectual também me proporcionou a oportunidade de crescimento pessoal. O Doutoramento trata-se, enigmaticamente, de um longo percurso individual e solitário, mas bem acompanhado. Por isso, deixo aqui o meu agradecimento a todos que, de uma forma ou de outra, me acompanharam e ajudaram a manter o rumo ao longo deste percurso.

À minha orientadora, Raquel Almeida, pela confiança que mais uma vez depositou em mim, dando-me a oportunidade de fazer o doutoramento no grupo e assim continuar o trabalho que desenvolvemos ao longo do meu mestrado. Obrigada pela disponibilidade, pelo entusiasmo, pela paciência, pelos conselhos, pelas reflexões e pela partilha de conhecimento. Agradeço imenso todas as oportunidades que me facultou pois contribuíram fortemente e positivamente para a Cientista que sou hoje. Muito obrigada.

À minha co-orientadora, Patrícia Mesquita, pela constante disponibilidade, otimismo, partilha de conhecimentos e discussões científicas. Pelos momentos hilariantes que só uma bioquímica poderia proporcionar a uma bióloga, especificamente a do APS, esta fica para a história. Obrigada pelas conversas e amizade.

Ao meu co-orientador, Filipe Pereira, pelo acompanhamento, disponibilidade e partilha de conhecimentos. Obrigada por me inspirar ao ser um excelente Cientista.

À minha tutora, Professora Fátima Gärtner, por ter aceitado acompanhar este meu percurso. Obrigada pela disponibilidade e constante simpatia.

Ao António Pombinho pela preciosa ajuda na descoberta dos nossos dois fármacos. Obrigada pela partilha de conhecimentos, pela dedicação e constante disponibilidade para discutir os resultados. Ao André Maia e à Mafalda Sousa por ter contribuído e facilitado a análise dos milhares de dados obtidos no high-throughput screening. À Paula Magalhães e à Tânia Meireles pela amizade, confiança e constante prontidão para resolver os desafios que surgiam na sala

dos vírus. À Raquel Lima e à Joana Peixoto pela partilha de conhecimentos e ajuda na realização do Seahorse assay.

À Rita Barros e à Ana Luísa Amaral que, apesar de já não fazerem parte do grupo há alguns anos, contribuíram positivamente para este meu percurso. Obrigada pela amizade e por todo o conhecimento partilhado. À Paulinha por ter sido a excelente aprendiz e uma das pessoas com quem mais gostei de partilhar a minha bancada do laboratório. Apesar de todas as falhas elétricas que sofriamos por tua causa, conseguimos encontrar o LINDO nas células. A tua passagem pelo grupo criou a grande amizade que temos hoje. À Nicole Pedro pelas conversas, gargalhadas, entreajuda e grande amizade. Obrigada por tudo, walkamos juntas!!

Ao meu *squad* pelo apoio e encorajamento que tanto me ajudaram a manter o rumo, motivação e a aliviar o stress ao longo deste percurso. Agradeço-vos também toda a compreensão relativa à minha ausência e adiamento de planos.

E por último, e mais importante, aos meus pais pelo amor, apoio incondicional, encorajamento, compreensão e paciência. Obrigada por me incentivarem a alcançar todos os meus objetivos, a lutar por eles sem nunca desistir, a ser persistente e determinada. Agradeço-vos também pela excelente vida que sempre me proporcionaram e que me permitiu chegar até aqui e continuar a fazer o que gosto. Muito obrigada por tudo, vocês são incríveis!!

ABSTRACT

Gastric cancer remains a serious health problem worldwide, with few therapeutic options. It is an extremely heterogeneous disease that presents diverse histopathological characteristics and molecular signatures. Cancer stem cells (CSCs) are believed to be involved in tumor initiation, progression, metastasis, recurrence, and therapy resistance, making them essential therapeutic targets. Still, the molecular circuits of CSCs that link stemness to therapy resistance and patient prognosis are not well understood. CSCs are generally identified by a combination of cell-surface markers that often differ between tumor types and whose robustness in distinguishing tumorigenic from non-tumorigenic cells is debatable. Therefore, ideal CSC markers are those that are required to maintain their stem-like characteristics, e.g., transcription factors (TFs). As several important stem cell TFs have been shown to be overexpressed in CSCs, fluorescence reporter systems activated by portions of promoters where these proteins bind have been developed to mark and track CSCs in various cancer types. SOX2 is recognized as a key pluripotency factor in multiple tissues and developmental stages. In cancer, SOX2 has been increasingly associated with the CSC phenotype and poor outcome of gastric cancer patients. Thus, the main objective of this thesis was to identify the transcriptional regulatory network in gastric CSCs, in order to harness it for therapeutic strategies and biomarker identification.

In this thesis, using the SORE6-GFP reporter system, which depends on SOX2 and/or OCT4 to drive GFP expression, we isolated and characterized a cancer stem-like cell subpopulation (SORE6+) from gastric cancer cell lines. Unlike SORE6- (non-CSCs), SORE6+ cells were enriched in several TFs, including SOX2, C-MYC, KLF4 and HMGA1, and exhibited characteristics of CSCs, such as asymmetric division (giving rise to GFP+ and GFP- cells), drug resistance, improved proliferation capacity and gastrosphere formation, and increased tumorigenic capacity *in vivo*. Through high-throughput drug screening, we discovered that monensin, a polyether ionophore antibiotic, can selectively target gastric CSCs, which may represent a valuable therapeutic weapon against gastric cancer. With our SORE6 cell model, we also sought to identify

compounds capable of converting SORE6⁻ into SORE6⁺ cells in order to disclose the molecular mechanisms involved in the characteristics of gastric CSCs. Thus, using the activation of the SORE6-GFP reporter system as a readout of successful cellular reprogramming, and a high-throughput drug screening, we uncovered that the iron chelator ciclopirox (CPX) can reprogram gastric non-CSCs into CSCs by inducing SOX2 expression. This ability of CPX appears to be comparable to that of the hypoxic mimetic agent CoCl₂ and seems to be associated with an increase in the expression of SOX2, C-MYC, HMGA1, KLF4 and HIF-1 α . Additionally, treatment with CPX or CoCl₂ induces a metabolic shift from OXPHOS to glycolysis, a potential pathway used by gastric CSCs to obtain energy, and also leads to cellular senescence, which has been associated with the properties of CSCs. We also discovered that a second exposure of cells to CPX increased the conversion efficiency of GFP⁻ cells into GFP⁺, showing that these cells, recovered from a first treatment, present greater cellular plasticity and resistance to CPX. We also observed that overexpression of HMGA1 in SORE6⁻ cells was able to generate some GFP⁺ cells by inducing SOX2 expression. We next compared SORE6⁻ HMGA1 GFP⁻ and SORE6⁻ HMGA1 GFP⁺ sorted cells and found that HMGA1 GFP⁺ cells exhibited stem-like characteristics and were enriched in stemness-related TFs. We also disclosed that HMGA1 expression facilitates CPX-mediated cellular reprogramming of gastric non-CSCs into CSCs. Finally, we evaluated the relevance of HMGA1 expression in gastric cancer and found that gastric cancer patients with high HMGA1 expression in the tumor benefit more from chemotherapy treatment than those with low expression.

Overall, the results presented in this thesis provided new information for a better understanding of the characteristics, behavior and impact of gastric CSCs on the development and treatment of gastric cancer. It also presented new gastric CSCs biomarkers and potential therapeutic agents.

RESUMO

O cancro do estômago continua a ser um grave problema de saúde em todo o mundo, com poucas opções terapêuticas. É uma doença extremamente heterogênea que apresenta diversas características histopatológicas e assinaturas moleculares. Acredita-se que as células estaminais de cancro (CSCs) estejam envolvidas na iniciação, progressão, metastização, recorrência e resistência à terapia do tumor, tornando-as alvos terapêuticos essenciais. No entanto, os circuitos moleculares das CSCs que ligam a estaminalidade à resistência à terapia e ao prognóstico do paciente não são bem compreendidos. As CSCs são geralmente identificadas através da combinação de marcadores de superfície celular que, muitas vezes, diferem entre os tipos de tumor e cuja robustez na distinção de células cancerígenas de não-cancerígenas é discutível. Assim, os marcadores ideais de CSC são aqueles que são necessários para a manutenção das características estaminais destas células, por exemplo, os fatores de transcrição (TFs). Como já foi demonstrado que vários TFs importantes de células estaminais estão sobreexpressos em CSCs, sistemas repórteres ativados por porções de promotores onde estas proteínas se ligam foram desenvolvidos para marcar e rastrear as CSCs em vários tipos de cancro. O SOX2 é um importante fator associado à pluripotência em vários tecidos e etapas de desenvolvimento. Em cancro, o SOX2 tem sido cada vez mais associado ao fenótipo de CSC e ao mau prognóstico de pacientes com cancro do estômago. Assim, o objetivo principal desta tese foi identificar a rede transcricional que regula as CSCs de estômago, de modo a usá-la para estratégias terapêuticas e identificação de biomarcadores.

Nesta tese, utilizando o sistema repórter SORE6-GFP, que depende da expressão de SOX2 e/ou OCT4 para conduzir a expressão de GFP, isolamos e caracterizamos uma subpopulação de CSCs (SORE6+) de linhas celulares de cancro do estômago. Ao contrário do SORE6- (não-CSCs), as células SORE6+ estão enriquecidas em vários TFs, incluindo SOX2, C-MYC, KLF4 e HMGA1, e exibem características de CSCs, tais como divisão assimétrica (dando origem a células GFP+ e GFP-), resistência a fármacos, melhor capacidade de proliferação e formação de gastroesfera e maior capacidade tumorigénica *in vivo*. Através da realização de uma triagem de alto rendimento de pequenas

moléculas descobrimos que a monensina, um antibiótico ionóforo poliéter, consegue atacar seletivamente as CSCs do estômago, o que pode representar uma valiosa arma terapêutica contra o cancro do estômago. Com o nosso modelo de células SORE6, também procuramos identificar compostos capazes de converter SORE6- em células SORE6+ de modo a revelar os mecanismos moleculares envolvidos nas características das CSCs do estômago. Assim, usando a ativação do sistema repórter SORE6-GFP como um indicador de reprogramação celular bem-sucedida, e uma triagem de alto rendimento de pequenas moléculas, descobrimos que o quelante de ferro ciclopirox (CPX) pode reprogramar não-CSCs do estômago em CSCs, através da indução da expressão de SOX2. Esta capacidade do CPX parece ser comparável à do agente mimético de hipoxia CoCl_2 , e parece estar associada a um aumento na expressão de SOX2, C-MYC, HMGA1, KLF4 e HIF-1 α . Além disso, o tratamento com CPX ou CoCl_2 induz uma alteração metabólica de OXPHOS para glicólise, uma via utilizada pelas CSCs do estômago para obter energia, e também leva à senescência celular, que tem sido associada às propriedades das CSCs. Descobrimos também que uma segunda exposição das células ao CPX aumentou a eficiência de conversão das células GFP- em GFP+, mostrando que estas células, recuperadas de um primeiro tratamento, apresentam maior plasticidade celular e resistência ao CPX. Observamos também que a sobreexpressão de HMGA1 nas células SORE6- foi capaz de gerar algumas células GFP+, através da indução da expressão de SOX2. Seguidamente, comparamos as células SORE6- HMGA1 GFP- e as SORE6- HMGA1 GFP+ e descobrimos que as células HMGA1 GFP+ exibem características de CSCs e estão enriquecidas em TFs relacionados com a estaminalidade. Também divulgamos que a expressão de HMGA1 facilita a reprogramação celular mediada por CPX de não-CSCs do estômago em CSCs. Por último, avaliamos a relevância da expressão de HMGA1 em cancro do estômago e descobrimos que pacientes com cancro do estômago com alta expressão de HMGA1 no tumor beneficiam mais da quimioterapia do que aqueles com baixa expressão deste fator de transcrição.

De forma geral, os resultados apresentados nesta tese fornecem novas informações para uma melhor compreensão das características, comportamento e impacto das CSCs no desenvolvimento e tratamento do cancro do estômago. Também apresenta novos biomarcadores de CSCs do estômago e potenciais agentes terapêuticos.

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FIGURES

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ABBREVIATIONS

5-FU: 5-Fluorouracil

ALDH: Aldehyde Dehydrogenase

ALDH1A1: Aldehyde Dehydrogenase 1 Family Member A1

ARNT2: Aryl Hydrocarbon Receptor Nuclear Translocator 2

AT-rich: High content of Adenine and Thymine residues

ATP: Adenosine Triphosphate

BAX: Bcl-2-associated X protein

BrdU: Bromodeoxyuridine (5'-bromo-2'-deoxyuridine)

C-JUN: Jun Proto-Oncogene protein, activator protein-1 (AP-1) transcription factor subunit

C-MYC: MYC Proto-Oncogene, bHLH transcription factor

CD133: Prominin 1

CD166: Activated Leukocyte Cell Adhesion Molecule (ALCAM)

CD184 (CXCR4): C-X-C Motif Chemokine Receptor 4

CD24: CD24 Molecule

CD44: CD44 Molecule (Indian Blood Group)

CD49f: Integrin Subunit Alpha 6 (ITGA6)

CD54: Intercellular Adhesion Molecule 1 (ICAM1)

CD71: Transferrin Receptor (TFRC)

CD90: Thy-1 Cell Surface Antigen (THY1)

CDKN1B: Cyclin-dependent kinase inhibitor 1B

cDNA: Complementary Deoxyribonucleic acid

CLPTM1L: Cleft lip and palate transmembrane protein 1-like

CNOT3: CCR4-NOT Transcription Complex Subunit 3

CoCl₂: Cobalt Chloride

CPX: Ciclopirox Olamine

CSC: Cancer Stem Cell

CSCs: Cancer Stem Cells

CTRL: negative control

CYP3A5: Cytochrome P450 Family 3 Subfamily A Member 5

DAB: 3,3'-Diaminobenzidine

DAPI: 4',6-Diamidino-2-phenylindole

DFS: Disease-free survival

DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DOX: Doxycycline
dsCopGFP: destabilized Green Fluorescent Protein
ECAR: Extracellular Acidification Rate
EDTA: Ethylenediaminetetraacetic acid
EpCAM: Epithelial Cell Adhesion Molecule
FACS: Fluorescence-Activated Cell Sorting
FOXA1: Forkhead Box A1
FOXA2: Forkhead Box A2
FOXC2: Forkhead Box C2
GATA6: GATA Binding Protein 6
GC: Gastric Cancer
GFP: Green Fluorescent Protein
GLI1: GLI Family Zinc Finger 1
GLI2: GLI Family Zinc Finger 2
H&E: Hematoxylin and Eosin
HER2: Human epidermal growth factor receptor-2
HES1: Hes Family BHLH Transcription Factor 1
HIF-1 α : Hypoxia inducible factor 1 subunit alpha
HMGA1: High Mobility group AT-hook 1
HMGA2: High Mobility group AT-hook 2
HOXA10: Homeobox A10
HOXA5: Homeobox A5
IC50: half-maximal inhibitory concentration
iPS: Induced pluripotent stem
ISL1: ISL LIM Homeobox 1
K⁺: Potassium ion
KLF4: Krüppel Like Factor 4
KLF5: Krüppel Like Factor 5
Lgr5: Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
LIN28: Lin-28 Homolog A
mCMVp: minimal cytomegalovirus promoter
MEIS2: Meis Homeobox 2
mRNA: messenger Ribonucleic acid
Na⁺: Sodium ion
Na₃VO₄: Sodium orthovanadate

NANOG: Nanog Homeobox
NOTCH1: Notch Receptor 1
OCR: Oxygen Consumption Rate
OCT1: POU Class 2 Homeobox 1
OCT4: POU Class 5 Homeobox 1
OLIG2: Oligodendrocyte Transcription Factor 2
OS: Overall survival
OXPHOS: Oxidative Phosphorylation
p-ERK: Phosphorylation of extracellular signal-regulated kinase
PARP: Poly(ADP-Ribose) Polymerase
PD-1: Programmed cell death protein 1
PI: Propidium Iodide
PMSF: Phenylmethylsulfonyl fluoride
POU3F2: POU Class 3 Homeobox 2
RARG: Retinoic Acid Receptor Gamma
RELA: RELA Proto-Oncogene, NF-KB Subunit
RNA: Ribonucleic acid
SA- β -gal: Senescence associated- β -galactosidase
SALL2: Spalt Like Transcription Factor 2
SASP: Senescence-associated secretory phenotype
SD: Standard deviation
siRNA: Small interfering RNA
SOD1: Superoxide Dismutase 1
SOX2: SRY-Box Transcription Factor 2
SOX4: SRY-Box Transcription Factor 4
SOX9: SRY-Box Transcription Factor 9
STAT3: Signal Transducer and Activator of Transcription 3
TCF4: Transcription Factor 4
TEAD2: TEA Domain Transcription Factor 2
TF: Transcription Factor
TFs: Transcription Factors
TNM: Tumor Node Metastasis
wt: wild-type
ZNF273: Zinc Finger Protein 273

CHAPTER I

GENERAL INTRODUCTION

The General Introduction contains parts and adaptations of the following publication:

Pádua, D., Figueira, P., Ribeiro, I., Almeida, R., & Mesquita, P. (2020). *The Relevance of Transcription Factors in Gastric and Colorectal Cancer Stem Cells Identification and Eradication*. *Frontiers in cell and developmental biology*, 8, 442. <https://doi.org/10.3389/fcell.2020.00442>

1. ANATOMY AND HISTOLOGY OF THE STOMACH

The stomach is an extremely important J-shaped organ of the digestive system, responsible for temporarily storing and digesting food (1). To aid digestion, the stomach secretes gastric acid, an acidic liquid composed of hydrochloric acid, mucus, and the digestive enzymes pepsin, lipase, and rennin (2). In addition, the stomach's acidic environment (pH 1.5 to 3.5) acts as a first line of defense against harmful pathogens (e.g., yeasts, bacteria, and viruses) (3, 4). The stomach absorbs very few substances such as water, monosaccharides (fructose and galactose) and disaccharides (lactose and sucrose). It can also absorb fat-soluble compounds and ethyl alcohol that cause gastric irritation, leading to the development of gastritis and gastric ulcers (2, 5).

Anatomically, the stomach has four main regions that can be distinguished through anatomical demarcations and histological differences (6). Cardia is the first region of the stomach; it is where food and liquids enter the stomach from the esophagus. Above and to the left of the cardia is the fundus, a dome-shaped region where the gases produced during digestion accumulate. Below the fundus is the body (corpus), the main and largest region of the stomach, where food is broken down and mixed. Following the body is the pylorus, the last region of the stomach that can be subdivided into two parts: the pyloric antrum which connects to the body and where digested food can be retained; and the pyloric canal which connects to the duodenum and from which stomach contents pass into the intestine (7).

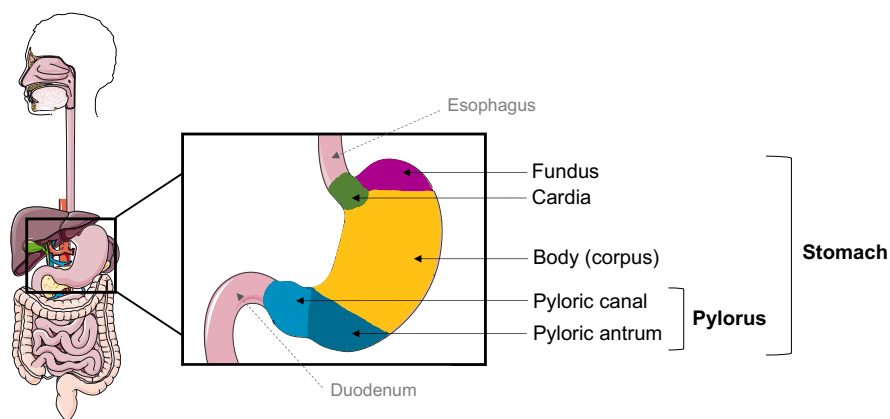


Figure 1 - Schematic representation of the regions of the stomach. A muscular sac-like organ divided into four main regions: cardia, fundus, body (corpus), and pylorus (pyloric antrum and pyloric canal). The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Histologically, the stomach wall has four tissue layers: mucosa, submucosa, muscularis and serosa (Figure 2) (7, 8). The mucosa, the inner layer, is formed by a layer of surface

epithelium and an underlying lamina propria and muscularis mucosae. The surface epithelium forms numerous small invaginations, also known as gastric pits, which are connected to the gastric glands, a branched tubular structures that extend through the lamina propria (Figure 2) (7). The cellular composition of the gastric glands differs according to their location and function. However, they contain highly proliferative gastric stem cells that are essential for the stomach epithelium regeneration and repair (Figure 2) (9). Separating the lamina propria from the submucosa layer is the muscularis mucosae, which consists of two thin sections of smooth muscle. The submucosa is a thick layer of loose connective tissue that contains blood and lymphatic vessels, and nerves. The muscularis layer is formed by smooth muscle and can be divided into three sublayers: oblique, circular and longitudinal (Figure 2). The serosa, the external layer, is composed of a thin mesothelium and connective tissues (7).

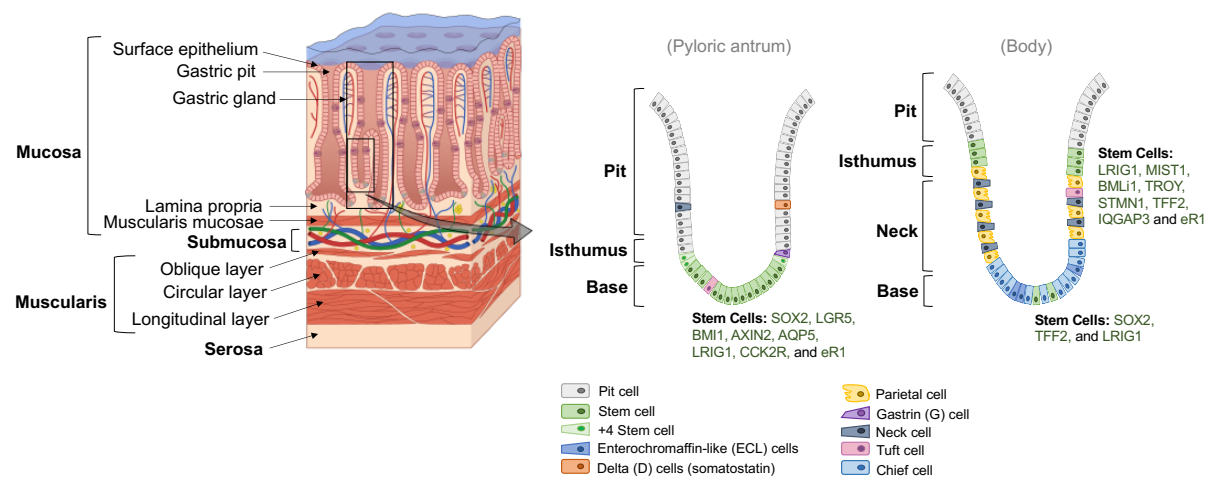


Figure 2 - Schematic representation of the histology of the stomach. Layers of the stomach wall; the mucosa (consists of loose connective tissue), the submucosa (consists of fibrous connective tissue), the muscularis (smooth muscle), the subserosa and serosa (consist of layers of connective tissue). Followed by the schematic illustration of the gastric glands and cell types from the pyloric antrum and body. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License and an image created by Collectivedogs, licensed under the Creative Commons Attribution-Share Alike 4.0 International license.

Overall, the stomach is a crucial organ of the digestive system responsible for digesting food and subsequently delivering the digested food to the duodenum. Although it is possible to survive without a stomach, this complex organ is essential for obtaining nutrients and energy from the food consumed, thus it is important to keep the stomach healthy (10, 11). However, hereditary and non-hereditary conditions and diseases can affect this organ, such

as gastritis, gastroparesis, gastroenteritis, peptic ulcers, non-ulcer dyspepsia and cancer (12). Stomach lesions and precancerous conditions such as metaplasia (when one cell type changes into another normal cell type as an adaptation to adverse environmental conditions (e.g., inflammation)) and dysplasia (early stage of neoplastic process), if not treated, confer a considerable risk for the development of gastric cancer (12, 13).

2. GASTRIC CANCER

Gastric cancer is a complex disease in which malignant cells arise in any region and layer of the stomach. If it arises in the cardia, it is called cardia gastric cancer, while if it arises in other regions of the stomach, it is called non-cardia gastric cancer (14).

2.1. Epidemiology and Risk Factors

Gastric cancer continues to represent a serious health problem and clinical challenge. It is the fifth most common cancer and the fourth leading cause of cancer-related deaths worldwide (15). Additionally, non-cardia gastric cancer is diagnosed twice as often as cardia gastric cancer (16). The incidence and mortality of gastric cancer vary significantly by geographic location and between women and men. East Asia, Eastern Europe and South America have the highest gastric cancer incidence and mortality rates, while South Asia, North and East Africa, North America, Australia and New Zealand have the lowest incidence and mortality rates (Figure 3) (15). Furthermore, age-standardized incidence and mortality rates are twice as high in men than in women (Figure 3) (15). Several studies suggest, among other factors, that exposure to estrogen reduces the risk of developing gastric cancer and therefore, as women have higher estrogen levels than men, they have greater protection against the disease (17-19). Focusing on Portugal, although the trend in incidence and mortality rates between men and women is the same as that observed worldwide, the death rate from gastric cancer is remarkably high (Figure 3) (15). Furthermore, there is great variation within the country, with incidence and mortality being much higher in the North of Portugal (20).

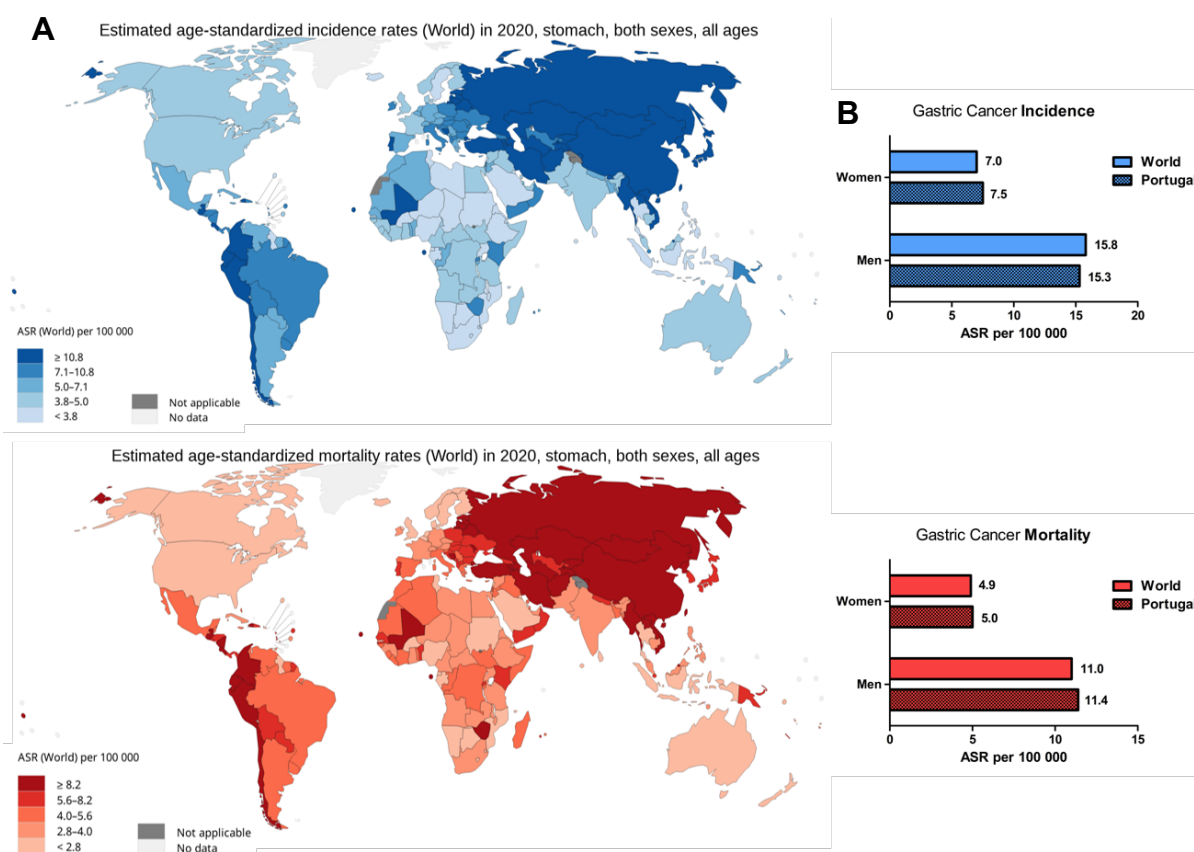


Figure 3 - Worldwide gastric cancer incidence and mortality rates (age-adjusted according to the world standard population (age-standardized rate (ASR)), per 100 000). **A)** Maps showing the worldwide distribution of gastric cancer incidence (blue) and mortality (red) in 2020. **B)** Comparison of gastric cancer incidence and mortality rates in men and women in Portugal and worldwide in 2020. Data source and Maps: GLOBOCAN 2020, international agency for research on cancer (IARC) (<http://gco.iarc.fr/today>), World Health Organization.

Gastric cancer is a multifactorial disease, resulting from a combination of environmental and behavioral factors, and genetic changes (21, 22). The main environmental factor that contributes to the onset of gastric cancer is, without a doubt, infection with the gram-negative bacteria *Helicobacter pylori* (classified as type I carcinogen by the IARC and WHO, since 1994) (23). *H. pylori* infection is the most common chronic bacterial infection in the world (Portugal has a high prevalence of *H. pylori* infection) and the leading cause of non-cardia gastric cancer (24-26). However, not all individuals infected with *H. pylori* develop gastric cancer, only 1 to 3% of infected individuals develop the disease (27). While it is not understood why this happens, it is believed to be related to the virulence of the strain, the susceptibility of the host, and the presence of other risk factors (28). Apart from *H. pylori* infection, gastric cancer has also been associated with the Epstein-Barr virus (EBV) (29, 30).

Poor dietary pattern represents a behavioral risk factor for the development of gastric cancer. A diet rich in industrially processed foods, red meat, smoked meat and fish, foods rich in saturated fat, salt and/or sugar, combined with low consumption of fruits and vegetables increases the risk of gastric cancer (31). Additionally, obesity also poses a potential risk for developing gastric cancer. Meta-analysis revealed that being overweight or obese was associated with the risk of cardia gastric cancer (32, 33). Another behavioral factor that also contributes to gastric cancer is tobacco smoking. A large systematic review and meta-analysis of 42 studies revealed that the risk of developing gastric cancer is 60% and 20% higher in men and women, respectively, compared to those who never smoked (34). Although some studies have been inconclusive, alcohol consumption has also been identified as a potential risk factor (35, 36). A systematic meta-analysis of 10 studies supported the hypothesis that alcohol consumption may increase the risk of developing gastric cancer (37).

Family history is another important risk factor for gastric cancer, although about 90% of cases of gastric cancer are sporadic and only around 10% are of familial origin (38). Of these 10%, approximately 3% result from hereditary diffuse gastric cancer (HDGC), whose main causes are germline mutations in the cadherin-1 (CDH1) gene (39). However, there are several cases in which no germline genetic abnormalities can be identified, making it impossible to implement the preventive measures that are recommended to patients with CDH1 mutation, such as prophylactic gastrectomy (40). Age is also a risk factor for gastric cancer, with most cases diagnosed after the age of 50 (41). However, in patients with hereditary or familial gastric cancer or HDGC, the disease appears at a relatively young age (< 40 years) (39).

Other risk factors, such as gastrointestinal microbiota, gastric ulcer, gastroesophageal reflux disease, exposure to ionizing radiation, blood group A and previous gastric surgery can also increase the risk of developing gastric cancer (42-47).

Thus, in some cases, gastric cancer can be prevented by reducing exposure to behavioral risk factors, through changes in lifestyle and dietary habits, while in other cases, especially in those of familial origin, a better knowledge of the disease is needed in order to reduce the incidence and mortality rates.

2.2. Histological and Molecular Classification

Gastric cancer exhibits marked histological heterogeneity both architecturally and cytologically. Most diagnosed cases of gastric cancer are adenocarcinomas (up to 95%) and, as mentioned earlier, they can be divided into cardia and non-cardia (48). Since 1965,

the Laurén classification of gastric cancer has been the most widely used and studied of all classification systems. Laurén classification divides gastric adenocarcinomas into two histological subtypes: diffuse and intestinal, in addition to the indeterminate type as an uncommon variant (49). Diffuse-type adenocarcinoma is poorly differentiated (cells do not look or behave like normal cells), with infiltration of non-cohesive tumor cells and metastasizes much more quickly than intestinal adenocarcinomas, leading to rapid disease progression and worse prognosis (48). It is more common in women and younger patients (50). Intestinal-type adenocarcinoma is the most frequent, generally occurs in men and at older ages (51). It is well differentiated (cells look and behave like normal cells), with cohesive neoplastic cells forming gland-like structures (48). Furthermore, some gastric cancers may have mixed features of diffuse and intestinal types (49). Although both types are associated with *H. pylori* infection, the intestinal type is the one with the greatest association with this infection and also with intestinal metaplasia of the gastric mucosa (52) while the diffuse type has been more associated with other risk factors (53). Another gastric cancer classification system was issued in 2010 by the World Health Organization (WHO) and updated in 2019 (54). The WHO classification is incredibly detailed and includes not only gastric adenocarcinoma, but also all other types of gastric neoplasms of lesser frequency. This classification subdivides gastric cancer into several histological patterns, including tubular, papillary, mucinous, mixed, and poorly cohesive (including signet ring cell carcinoma and non-signet ring cell carcinoma), as well as uncommon histological variants (54). It is also possible to draw parallels between the two classification systems. Tubular and papillary gastric adenocarcinoma of the WHO classification system corresponds to intestinal gastric adenocarcinoma of the Laurén classification (55). Diffuse gastric adenocarcinoma of the Laurén classification corresponds to poorly cohesive gastric adenocarcinoma (including signet ring cell carcinoma) (55). Briefly, gastric tubular adenocarcinoma is the most common and is characterized by prominent dilated or slit-like tubules that invade the muscle layer (56). Papillary gastric adenocarcinoma tends to affect older people and is characterized by elongated finger-like epithelial projections with cores of fibrovascular connective tissue. Poorly cohesive gastric adenocarcinoma, if it is signet ring cell type, it is composed mainly of signet ring cells, while poorly cohesive non-signet ring cells type is morphologically similar to histiocytes, lymphocytes, and plasma cells (57).

In addition to being a histologically heterogeneous disease, gastric cancer also presents genetic and molecular heterogeneity. Throughout the carcinogenic process, there is an accumulation of several genetic and molecular abnormalities, including the activation of oncogenes and inactivation of tumor suppressor genes, point mutations, overexpression of growth factors and DNA repair genes (58, 59). Therefore, the molecular landscape of gastric cancer has also been explored and used to classify this pathology in order to improve

targeted treatment. In 2013, Lei et al, when comparing patterns of gene expression among 248 gastric malignant tumors, classified gastric cancer into three subtypes with distinct molecular and genetic characteristics and also different response to therapy: proliferative, metabolic and mesenchymal (60). However, in 2014, the Cancer Genome Atlas Research Network (TCGA) performed a broad molecular characterization of gastric cancer based on the evaluation of 295 primary gastric adenocarcinomas using sequencing data from six molecular platforms (61). The results of this analysis allowed classifying gastric cancer into four molecular subtypes: chromosomal instability subtypes (CIN), EBV-positive tumors, microsatellite instability-high status (MSI) and genomically stable (GS) (61). This study also had a significant impact on the development of guidelines for patient stratification and trials of targeted therapies (62). In addition, the Asian Cancer Research Group (ACRG) also proposed a gastric cancer molecular classification based on the analysis of 300 gastric cancer cases. The ACRG also proposed four molecular subtypes: MSI, microsatellite stable (MSS)/EMT, MSS/TP53 mutant (TP53+) and MSS/TP53 wild-type (TP53-) (63).

2.3. Staging, Diagnosis and Treatment

Cancer stage is the most significant prognostic factor for gastric cancer. It provides information about the size of the tumor, whether it has spread, and how far it has spread from where it formed (64). Thus, the stomach layers are essential for assessing the stage of the disease. As the cancer grows from the mucosa to the serosa, the stage becomes more advanced, and the prognosis becomes poorer. The Tumor Node Metastasis (TNM) system is the most widely used cancer staging system worldwide. It is based on the size and extent of the primary tumor (T), regional lymph nodes involvement (N) and the presence or absence of metastatic disease (M) (65). The initial stage corresponds to stage 0 (carcinoma *in situ*) and then varies from stages I to IV (Table 1) (65). The lower the number, the less the cancer has spread and better the prognosis tends to be.

Table 1 - TNM staging system for gastric cancer. Each stage has a number from 0 to IV. Stage I is subdivided into IA and IB, stage II is subdivided into IIA and IIB and stage III is subdivided into IIIA, IIIB and IIIC. The higher the number, the more the cancer has spread. Adapted from the 8th edition of the American Joint Committee on Cancer tumor-node-metastasis staging system for gastric cancer (2018) (65).

Stage	Subdivision	Description and Stage grouping
0	---	Carcinoma <i>in situ</i> . - Abnormal cells found in the mucosa (high grade dysplasia) or cancer cells only present in the top layer of cells of the mucosa (innermost layer of the stomach) that have not grown into deeper layers of tissue such as the lamina propria (Tis); Cancer has not spread to nearby lymph nodes (N0); Cancer has not spread to distant parts of the body (M0).

I	IA	- The main tumor has grown from the top layer of cells of the mucosa to the next layers below (lamina propria, the muscularis mucosa, or submucosa) (T1); N0 ; M0 .
	IB	- T1 ; Cancer has spread to 1 to 2 nearby lymph nodes (N1); M0 . OR - The main tumor is growing into the muscularis propria layer (T2); N0 ; M0 .
II	IIA	- T1 ; Cancer has spread to 3 to 6 nearby lymph nodes (N2); M0 . OR - T2 ; N1 ; M0 . OR - The main tumor is growing into the subserosa layer (T3); N0 ; M0 .
	IIB	- T1 ; Cancer has spread to 7 to 15 nearby lymph nodes (N3a); M0 . OR - T2 ; N2 ; M0 . OR - T3 ; N1 ; M0 . OR - The main tumor has grown through the stomach wall into the serosa, but it hasn't grown into any of the nearby organs or structures (T4a); N0 ; M0 .
III	IIIA	- T2 ; N3a ; M0 . OR - T3 ; N2 ; M0 . OR - T4a ; N1 ; M0 . OR - T4a ; N2 ; M0 . OR - The main tumor has grown through the stomach wall and into nearby organs or structures (T4b); N0 ; M0 .
	IIIB	- T1 ; Cancer has spread to 16 or more nearby lymph nodes (N3b); M0 . OR - T2 ; N3b ; M0 . OR - T3 ; N3a ; M0 . OR - T4a ; N3a ; M0 . OR - T4b ; N2 ; M0 .
	IIIC	- T3 ; N3b ; M0 . OR - T4a ; N3b ; M0 . OR - T4b ; N3a ; M0 . OR - T4b ; N3b ; M0 .
IV	---	- Cancer might or might not have grown into any of the layers of the stomach wall (Any T); Cancer might or might not have spread to nearby lymph nodes (Any N); Cancer has spread to distant organs such as the liver, lungs, brain, or the peritoneum (the lining of the space around the digestive organs) (M1).

Gastric cancer grows slowly over several years without causing noticeable symptoms. Thus, the diagnosis of gastric cancer is usually made at a later stage, a factor that contributes to the high mortality rate of the disease. The diagnosis of this pathology can be carried out through several tests, with endoscopic examination and forceps biopsy being the gold standard method (62). Multiple biopsies are performed to provide material of adequate size for histological and molecular analyses (62). To identify the stage of the disease, several procedures can be performed, including endoscopic ultrasound also called endosonography (allows identification of infiltrated regions of the gastric wall), endoscopic mucosal resection and endoscopic submucosal dissection (62). In addition, other procedures can be used, such as computed tomography (CT) of the chest, which may include the pelvis, laparoscopy plus washings and positron emission tomography (PET) – CT scan (62).

Distinct types of treatments are available for gastric cancer. Many factors are considered, such as the stage of the cancer and the patient's overall health, when considering a specific treatment. Surgical resection is the best option for operable gastric cancer. It is potentially curative for stage I, however, some patients relapse, so combined therapies are standard for stage $\geq$ IB (62). Surgery can also be performed in stages II and III, but radiotherapy and chemotherapy (perioperative, neoadjuvant and/or adjuvant) are necessary to improve patients' overall survival. Chemotherapy for gastric cancer includes several drugs such as capecitabine (an antimetabolite used when the cancer is HER2 positive), cisplatin (an alkylating agent that damages the DNA of dividing cells, leading to the cessation of tumor growth), docetaxel (a semisynthetic taxane that blocks cell division by interfering with microtubule polymerization dynamics), doxorubicin (an anthracycline that targets topoisomerase II), epirubicin (an anthracycline that is an inhibitor of topoisomerase II), irinotecan (an inhibitor of topoisomerase I), leucovorin (a folic acid analogue used in combination with other chemotherapy drugs to increase their cytotoxic effect and/or decrease their harmful side effects), oxaliplatin (an alkylating agent that causes DNA damage through DNA cross-linking), paclitaxel (a taxane that targets microtubules), the trifluridine/tipiracil combination (trifluridine stops cell division by incorporating itself into the DNA strain, while tipiracil slows the degradation rate of trifluridine) and 5-fluorouracil (5-FU) (62). In particular, the antimetabolite 5-FU is an uracil analogue with a fluorine atom at the C-5 position (66). The cytotoxic effect of 5-FU is induced through the inhibition of thymidylate synthase (TS) and the incorporation of its metabolites into DNA and RNA (67). Chemotherapy with 5-FU is the first treatment of choice for advanced gastric cancer. It is a key component of several chemotherapy regimens, including FOLFOX (leucovorin plus 5-FU and oxaliplatin), one of the most commonly used regimens in the first-line treatment of metastatic gastric cancer, and the adjuvant chemotherapy regimen XELOX (capecitabine

(a 5-FU prodrug) plus oxaliplatin) (68). Thus, for stage IV, when the cancer cannot be removed and/or has metastasized, the main treatment option is chemotherapy with double or triple combinations of platinum/fluoropyrimidine or capecitabine as the main treatment (62, 69). Other forms and combinations of treatment have also been used as targeted therapy, including immunotherapy. This type of precision medicine can be used alone or in combination with chemotherapy in order to improve the ability of the body's immune system to recognize and eliminate cancer cells. However, not all patients can benefit from immunotherapy, only patients with specific molecular signatures, such as expression of programmed cell death protein 1 (PD-1) and HER2, will be suitable candidates. (70). Gastric cancer immunotherapy uses monoclonal antibodies (nivolumab and pembrolizumab) to target, and block programmed PD-1 (62). Nivolumab is used together with a fluoropyrimidine and platinum chemotherapy, while pembrolizumab is used with trastuzumab (a HER2 inhibitor) and fluoropyrimidine and platinum chemotherapy in patients whose cancer is HER2 positive and has spread or cannot be removed surgically (62). Targeted therapy with ramucirumab (an angiogenesis inhibitor), regorafenib (a kinase inhibitor), trastuzumab or trastuzumab deruxtecan (an antibody–drug conjugate that inhibits HER2 and blocks topoisomerase I) can also be applied in advanced gastric cancer or those with metastasis (62).

However, cancer heterogeneity at molecular, histological and phenotypic levels plays a vital role in therapeutic resistance and tumor recurrence, with cancer stem cells (CSCs) being among the main factors causing cancer heterogeneity and treatment failure (71, 72).

3. CANCER STEM CELLS

The CSC model of tumor progression hypothesizes that a small subpopulation of cancer cells that display stem-like properties sustains tumor growth, metastasis, relapse and resistance to chemotherapy (71, 73). CSCs can undergo symmetric and asymmetric divisions, having the ability to give rise to all the distinct types of cancer cells within the tumor (74). However, the origin of CSCs is still unclear and controversial. Various hypotheses suggest that depending on the tumor type, CSCs might be derived from adult stem cells, adult progenitor cells that underwent mutations, or differentiated cells that gained stem-like properties through dedifferentiation (75). The first evidence for the existence of CSCs was reported in acute myeloid leukemia (AML), where a small population of CD34+/CD38- AML cells showed the ability to initiate tumor growth after transplantation into immunodeficient mice, establishing the hierarchical organization of AML (76). Following this study, others also reported the existence of CSCs in several types of cancer, including

gastric cancer (77, 78). Numerous studies have demonstrated that CSCs share biomarkers with normal stem cells, so specific markers for their identification have been explored over the years (79).

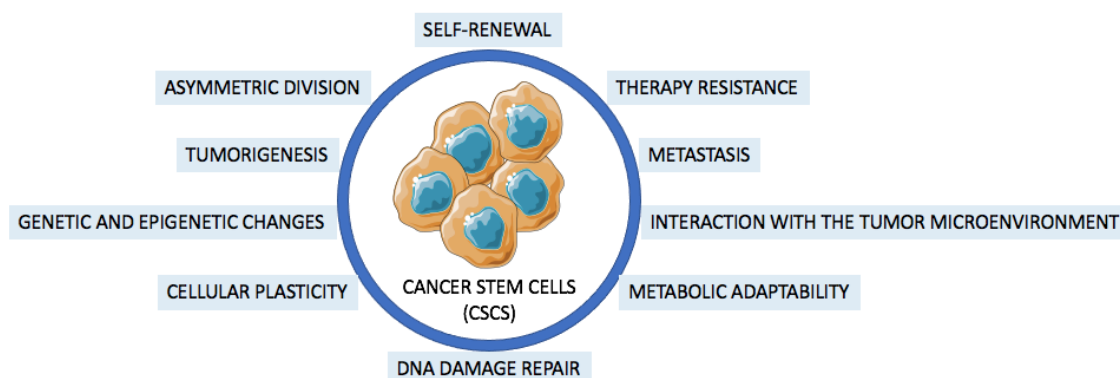


Figure 4 – Cancer stem cells (CSCs) features. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

3.1. Identification Challenges

Identification of normal stem cells is now a straightforward process due to their well-recognized set of biomarkers, whereas identification of CSCs is a challenging task resulting from their complex phenotype, which differs from one tumor to another (80). Furthermore, CSCs represent a small percentage of tumor cells within the total tumor mass, making their detection even more difficult in heterogeneous tumors. The use of different combinations of cell-surface markers has been the main strategy to identify CSCs in distinct types of tumors (81). Cell-surface markers are chosen according to their expression and relevance to particular tumor type, allowing the distinction of CSCs from non-CSCs (80). In addition to cell-surface markers the intracellular protein aldehyde dehydrogenase I (ALDH1) has also been utilized to pinpoint CSCs (82). In gastric cancer, the list of biomarkers capable of identifying CSCs is growing (Table 2). This means that interest in identifying and targeting gastric CSCs is increasing, but it also means that markers already in use are not uniformly advantageous for detecting CSCs. In particular, inconsistencies remain about which cell-surface marker might be the ideal marker to identify gastric CSCs from cell lines and primary tumors.

Table 2 - Cancer stem cell (CSC) biomarkers for gastric cancer. The table contain the most reported gastric CSCs markers and the characteristics exhibited by CSCs identified based on these biomarkers. Adapted from (83).

CSCs biomarkers	Implication	References
CD44	Tumorigenicity, self-renewal and drug resistance	(78, 84-86)
CD44 combined with CD24	Tumorigenicity and self-renewal	(87)
CD44 combined with CD54	Tumorigenicity, self-renewal and hierarchical organization	(88)
CD44 combined with EpCAM	Tumorigenicity, drug resistance and tumor heterogeneity	(89)
CD44 combined with CD24, CD54 and EpCAM	Tumorigenicity	(90)
CD49f	Tumorigenicity and drug resistance	(91)
CD71	Tumorigenicity, self-renewal, cell invasion and drug resistance	(92)
CD90	Tumorigenicity and self-renewal	(93)
CD133	Poorly differentiated; independent prognostic factor	(85, 86, 94-96)
CD133 combined with CD166	Self-renewal, cell invasion and drug resistance	(97)
CD184 (CXCR4)	Tumorigenicity and drug resistance	(98)
Lgr5	Tumorigenicity	(99)
ALDH	Tumorigenicity, self-renewal, drug resistance and tumor heterogeneity	(86, 100)

3.2. Gastric Cancer Stem Cells

Several biomarkers have emerged for gastric CSC identification. The transmembrane glycoprotein CD44 was the first described cell-surface marker used in gastric CSC identification. Takaishi et al. and his collaborators analyzed a panel of six gastric cancer cell lines and in three of them – NCI-N87, MKN-45 and MKN-74 – a small CD44+ cell subpopulation displayed CSC features such as self-renewal, asymmetric division, spheroid colony formation, and *in vivo* tumorigenic ability. They also observed that the CD44+ subpopulation had a higher resistance to anticancer drugs when compared to CD44– cells. However, in the other three cell lines – AGS, Kato III and MKN28 – the CD44 cell-surface marker was not able to mark cells with stem-like properties (78). Clinically, CD44+ cancer

cells at the invasive gastric cancer front are associated with poor patient survival (85, 101). Later, Zhang et al. combined CD44 with CD24, a signal transducer, and successfully detected a CD44+CD24+ cellular subpopulation with CSCs characteristics, such as the capability to self-renew and to originate differentiated progeny. Additionally, they showed that CD44+CD24+ cells had higher ability to form tumors when injected into immunodeficient mice, compared to the CD44–CD24– cells (87). The CD54 cell-surface marker, also known as ICAM-1 (intercellular adhesion molecule 1), was combined with CD44 to isolate gastric CSCs from tumor tissues and peripheral blood of patients with gastric cancer. The CD44+CD54+ cells exhibited *in vitro* and *in vivo* self-renewal ability, formed gastric tumorspheres and originated tumors similar to the original human tumor when injected into immunodeficient mice (88). The epithelial cell adhesion molecule (EpCAM) has also been used in combination with CD44 to mark gastric CSCs. The small EpCAM+/CD44+ subpopulation isolated from primary human gastric cancer tissues was more resistant to anticancer drugs including 5-FU, doxorubicin and paclitaxel, when compared with EpCAM+/CD44–, EpCAM–/CD44+ and EpCAM–/CD44– cells (89, 102). It also showed the capacity to form sphere-like structures in serum free conditions and greater ability to originate tumors in immunocompromised mice (89). The tumors formed after inoculation of the EpCAM+/CD44+ cells recapitulated the heterogeneous morphology and phenotype present in the original gastric tumor (89). Gómez-Gallegos et al. used CD44 combined with CD24, CD54 and EpCAM to identify gastric CSCs. Their work suggested that CD44+/CD24+/CD54+/EpCAM+ cells from patient tissue samples were associated with metastatic disease (90). Fukamachi et al. identified another potential gastric CSC marker, the CD49f, an integrin $\alpha 6$ (ITGA6) that is a subunit of laminin receptors. Their work showed that CD49f+ gastric cancer cells originated tumors when subcutaneously injected into immunodeficient mice, while CD49f– cells did not. They also demonstrated that some of the CD49f+ sphere-forming cells were more resistant to doxorubicin, 5-FU and doxifluridine (a 5-FU prodrug) than the other gastric cancer cells studied (91). Another cell-surface marker identified as a gastric CSC marker is the CD71 transferrin receptor. In this case, it was demonstrated that the CD71– subpopulation from the MKN-1 gastric cancer cell line displayed CSC features, contrary to CD71+ cells. The CD71– cells were more resistant to 5-FU than CD71+, had higher tumorigenic ability and were mostly present in the invasive front of the tumor (92). The cell-surface glycoprotein CD90 (Thy-1) appeared as a potential gastric CSC marker since it was capable of identifying a small population with *in vivo* tumorigenic and self-renewal ability. Additionally, 25% of the gastric primary tumors possessed a higher expression of HER2, which was correlated with the higher expression of CD90 (93). CD133 (prominin-1), a pentaspan transmembrane glycoprotein, is described as a gastric CSC marker due to the fact that its expression is positively correlated with tumor

aggressiveness in gastric cancer patients (85, 86, 94, 95, 103). Zhao et al. showed that the frequency of CD133+ in gastric primary tumors samples was higher than CD133– cells and CD133 was associated with poor prognosis in gastric cancer (104). Also, spheroid cells from gastric cancer cell lines and primary gastric cancer tissues presented CD133 expression and displayed several features of CSCs (105). Ni et al. combined CD133 with CD166 to mark gastric CSCs, showing that CD133+/CD166+ cell subpopulations from BGC-823 and SGC-790 gastric cancer cell lines have CSCs characteristics and are enriched in OCT4 and C-MYC (97). New markers have emerged in the study of gastric CSCs and demonstrated to be able to mark a small population with stem-like features, specifically CXCR4 (C-X-C chemokine receptor type 4) also known as CD184 and Lgr5 (leucine-rich repeat-containing G-protein coupled receptor 5) (98, 99). Also, the intracellular enzyme aldehyde dehydrogenase (ALDH) has been used to identify gastric CSCs. Zhi et al. were able to divide NCI-N87 and SNU-1 gastric cancer cell lines into ALDH+ and ALDH– cells. The ALDH+ cells presented CSC features such as higher levels of SOX2, NANOG and Nestin, formed more sphere-like structures and had higher resistance to 5-FU and cisplatin (100).

3.2.1. Transcription Factors as Potential Biomarkers

One of the challenges in targeting CSCs is identifying specific markers associated with their phenotype. It is known that normal stem cells and CSCs share core stemness signaling pathways such as Notch, Hedgehog, WNT/ β -catenin and JAK/STAT, that have pivotal roles in maintaining stem cell properties and regulating their transcriptional program (106). SOX2, OCT4, KLF4, C-MYC, HMGA1, HIF-1 α and NANOG are some of the key transcription factors (TFs) known to promote stemness by upregulating genes involved in self-renewal and pluripotency, while suppressing genes involved in differentiation (107-110). Key stem cell TFs like SOX2, OCT4, and NANOG have been proven to be overexpressed in CSCs (111-115). For that reason, fluorescence reporter systems driven by portions of promoters where these proteins bind have been developed to allow CSCs to be labeled and tracked in real-time. These reporter systems appear to be a powerful tool to identify and study CSCs more efficiently than cell-surface markers (116). Tang et al. developed a flexible lentiviral-based reporter system (SORE6-GFP) that allows direct and in real-time visualization of CSCs based on SOX2/OCT4 expression (114). This reporter system consists of six tandem repeats of a composite SOX2/OCT4 response element (SORE6) coupled to a minimal cytomegalovirus promoter (mCMVp) that drives GFP expression (Figure 5A). Thus, GFP expression only occurs if cells express sufficient levels of SOX2 and/or OCT4 (Figure 5B). By using this reporter system, Tang et al. were able to isolate and characterize a small

population of breast cancer cells with cancer stem-like properties, such as self-renewal, asymmetric division, drug resistance, and the ability to initiate tumors and metastasis *in vivo* (114).

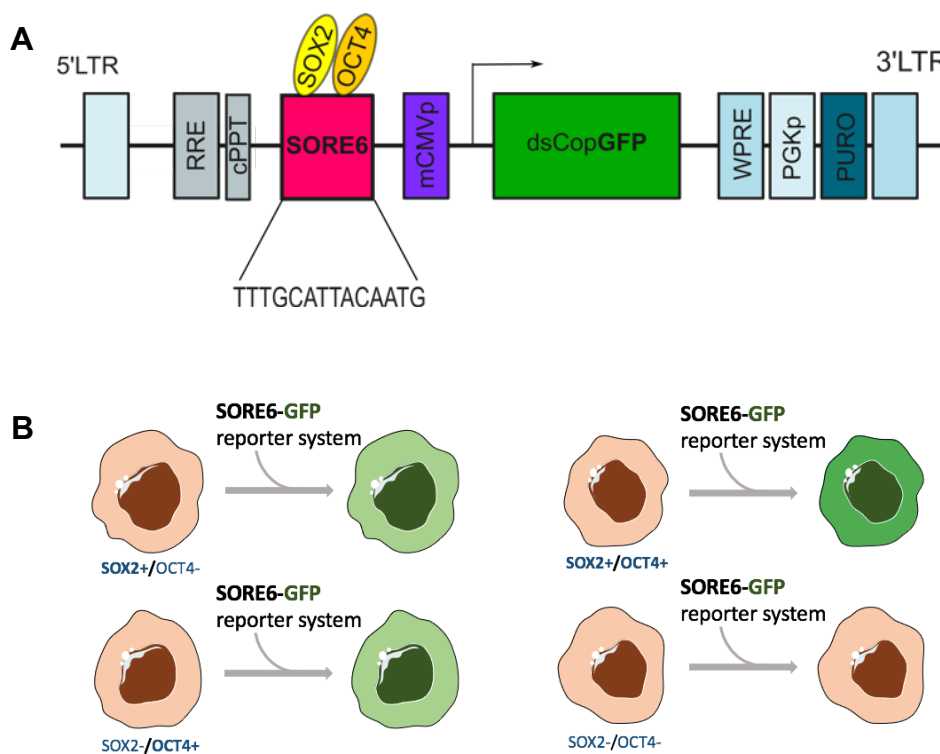


Figure 5 – The lentiviral SORE6-GFP reported system marks a small cell population enriched in SOX2 and/or OCT4 expression. A) Schematic representation of the lentiviral SORE6-GFP reporter system. SORE6 is the SOX2/OCT4 composite response element and drives the destabilized fluorescent protein (dsCopGFP) (114). **B)** If transduced cells express sufficient levels of SOX2 and/or OCT4, the SORE6-GFP reporter system will be activated, and the cells will become GFP-positive. The intensity of GFP expression is associated with the expression levels of SOX2 and/or OCT4. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Using the same principle, other authors have been developing similar systems to identify and characterize CSCs in a variety of solid tumors. Buczek et al. established a method using a lentiviral construct carrying the promoter of NANOG to identify prostate CSCs and more recently, Ghanei et al. introduced a similar approach for the isolation of CSCs in a breast cancer cell line based on the single expression of OCT4 (115, 117). Although the use of cell-surface markers is the most trusted approach to detect these cells, several studies demonstrated that they lack specificity and cannot be used for real time assessment of CSCs behavior, which could give new insights about their properties and possible targets (114). Growing evidences support that specific TFs overexpressed in

normal gastrointestinal stem cells may contribute to the self-renewal characteristics of CSCs in gastric cancer and are related with patient prognosis (118, 119). This makes them a powerful tool in CSCs identification and study.

3.2.1.1. SOX2 (SRY-Box Transcription Factor 2)

SOX2 is a master regulator that belongs to the family of high-mobility group TFs. It plays many roles throughout development and cell differentiation in normal tissues, namely in mammalian embryogenesis, morphogenesis and homeostasis of the foregut-derived epithelia of the esophagus, lung and trachea (120, 121). SOX2's role in stemness was strengthened with Takahashi and Yamanaka's findings since they were able to reprogram mouse embryonic fibroblasts into induced pluripotent stem (iPS) cells, by introducing SOX2 along with OCT3/4, C-MYC and KLF4 (122). In cancer, SOX2 has increasingly been associated with a CSC phenotype in several tumors (123-125). However, the role of SOX2 in gastric cancer is controversial because some authors report that high SOX2 expression is linked to a better gastric cancer prognosis, while other authors associate high SOX2 expression with a more aggressive phenotype, worse prognosis and worse response to therapy (105, 123, 126-133). Wang et al. reported that SOX2 expression inhibited gastric cancer cell proliferation and metastasis and promoted apoptosis (129). On the contrary, Hütz et al. observed that inhibition of SOX2 resulted in reduced cell proliferation and migration, increased apoptosis, changes in cell cycle and reduced tumorigenic potential of cells *in vivo* (126). Comparable results were observed *in vivo*, where suppression of SOX2 resulted in reduced tumor growth, decreased tumorigenicity and chemoresistance (134). Taken together, these findings indicate that SOX2 has a critical role in several aspects of CSCs biology.

3.2.1.2. OCT4 (POU Class 5 Homeobox 1)

OCT4, a homeodomain TF of the Pit-Oct-Unc family, is expressed in embryonic stem cells, where it maintains stem cell-like properties, and in adult stem cells, being involved in their proliferation and differentiation (Loh et al., 2006; Han et al., 2014). Abnormal expression of OCT4 has been observed in different tumor types, including gastric cancer (132, 135). Studies have shown that its expression is positively correlated with more aggressive and metastatic tumors, as well as with poorer overall prognosis (105, 132). Chen and collaborators demonstrated that overexpression of OCT4 in gastric cancer cells led differentiated cancer cells to become undifferentiated and acquiring self-renewal capacity

and another study reported that downregulation of OCT4 induced differentiation in gastric cancer cells (136, 137). Additionally, Zhang X. et al. demonstrated a p-ERK mediated positive feedback loop between the cell surface marker CD44 and OCT4, which is responsible for sustaining gastric CSCs properties (105).

3.2.1.3. KLF4 (Krüppel Like Factor 4)

KLF4, also known as the gut-enriched Krüppel like factor (GKLF), is strongly expressed in post-mitotic and terminally differentiated epithelial tissues, along with those of the gastrointestinal tract (138, 139). It is suggested that it may have an anticancer role in gastric cancer, being downregulated due to hypermethylation and loss of heterozygosity in gastric CSCs (138). Several studies have shown that low expression of KLF4 is negatively associated with patient overall survival and may be a useful prognostic marker in gastric cancer patients (140-144). It has been reported to be enriched in CSCs from breast tumors, however, little is known about gastric CSCs (145, 146).

3.2.1.4. C-MYC (MYC Proto-Oncogene, bHLH Transcription Factor)

C-MYC is an essential TF that regulates genes that take part in biological processes such as self-renewal, differentiation, growth and metabolism (147, 148). Although it is one of the most commonly activated oncogenes involved in the pathogenesis of cancer, its overexpression alone is unable to induce the transformation of normal cells into tumor cells (149). The role of C-MYC in gastric cancer is less studied than in other tumor types. It has been suggested as a CSC marker in some tumors such as small-cell lung cancer, prostate cancer, neuroblastoma, glioblastoma and hematopoietic malignancies, but the expression and relevance in gastric cancer has not yet been clarified (149). Some authors associate C-MYC deregulation with poor prognostic features (150-152). Additionally, C-MYC is positively correlated with pluripotency markers such as SOX2, OCT4, and NANOG (153).

3.2.1.5. HMGA1 (high mobility group AT-hook 1)

HMGA1 is a non-histone chromatin structural protein involved in the regulation of gene expression. It can alter the structure of DNA by binding to the minor groove of AT-rich regions and promotes the interaction between transcription regulatory proteins. HMGA1 is highly expressed during embryogenesis and very poorly expressed in adults. However, its expression has been shown to be elevated in several types of cancer (154). In particular,

HMGA1 was first discovered in highly proliferative and widely metastatic HeLa cervical cancer cells (155). The relevance of HMGA1 expression in gastric cancer was little explored. Nam et al. showed that its expression is associated with the gastric malignant phenotype (156). Cao et al. also reported that highly expressed HMGA1 predicts a poor clinical outcome in gastric cancer patients (157). Furthermore, HMGA1 maintains cell proliferation, promoting the growth and metastasis of gastric cancer (158, 159). HMGA1 has also been shown to be expressed in CSCs and promote the phenotype of CSCs. It positively regulates pluripotency genes, inducing the expression of important TFs, such as SOX2, OCT4 and NANOG (108). However, little is known about the role of HMGA1 in gastric CSCs.

3.2.1.6. HIF-1 α (hypoxia inducible factor 1 subunit alpha)

HIF-1 α is a master transcriptional regulator of the adaptive response to hypoxia (a state of reduced partial pressure of oxygen in the cell). Under hypoxic conditions, enzymatic inhibition of prolyl hydroxylase (PHDs) occurs, therefore, there is no recognition of HIF-1 α by the von Hippel-Lindau protein (VHL) or proteasomal degradation, leading to the consequent stabilization of HIF-1 α (160). Thus, the HIF-1 α protein undergoes translocation and accumulation in the nucleus, where it will dimerize with the HIF-1 β subunits and subsequently form the HIF complex (161, 162). HIF-1 α expression can also be induced through chemical hypoxia using the hypoxia mimetic mediator cobalt chloride (CoCl₂) (163). HIF-1 α is often overexpressed in gastric cancer and associated with drug resistance and poor overall survival in gastric cancer patients (164-166). In particular, 5-FU treatment may cause an increase of HIF-1 α expression, which would eventually result in resistance to this drug (167). HIF-1 α is differentially expressed between CSCs and non-CSCs (168). Furthermore, HIF induces the expression of several TFs including SOX2, OCT4, C-MYC, NANOG and KLF4 (169). Luo et al. and Miao et al. reported that HIF-1 α promotes the stemness of gastric cancer cells (170, 171). However, more research is needed to evaluate the role of HIF-1 α in gastric CSCs.

3.2.1.7. NANOG (Nanog Homeobox)

NANOG was first discovered in embryonic stem cells and is a key TF involved in self-renewal and pluripotency (172). It is typically silenced in normal somatic cells, although abnormal expression has been reported in malignant tumors such as gastric cancer (118, 173). Previous studies demonstrate that NANOG is highly expressed in gastric cancer and

significantly associated with tumor size and grade, along with decreased overall survival (132, 174). Although studies indicate that NANOG is associated with a CSC phenotype, it remains unclear its role in gastric CSCs maintenance (146, 174). Recently, studies have shown that NANOGP8, one of the pseudogenes of the NANOG gene family, is a key regulator of gastric CSCs, being associated with cell proliferation, migration, invasion and drug resistance (175, 176).

3.2.2. Interaction with the Tumor Microenvironment

The close interaction between CSCs and their niche is fundamental for maintaining the stemness of CSCs and tumor progression. The CSC niche, a specific tumor microenvironment, which consists of stroma, micro-vessels, hypoxic regions, cancer-associated fibroblasts (CAFs), cancer-associated mesenchymal stem cells (MSCs), tumor-associated macrophages (TAMs) and extracellular matrix, secretes soluble factors (e.g., cytokines and growth factors) that are necessary for cancer cell survival (149, 177, 178). Growth factors and cytokines regulate Wnt, Notch, JAK-STAT3 and other signaling pathways thereby stimulating growth, epithelial-to-mesenchymal transition (EMT), invasion, angiogenesis, metastasis and inhibiting apoptosis (149). These pathways are required for the self-renewal and maintenance of CSCs. For instance, hypoxia, which also maintains a stem-like phenotype in gastric cancer through the increased expression of HIF-1 α and HIF-2 α , maintains the Wnt/ β -catenin signaling pathway and interacts/activates stemness-related TFs such as SOX2 and OCT4 (165, 179-183). Cancer cells also secrete growth factors and proteases to promote changes in their microenvironment (184). Additionally, cancer cells mediate the production of inflammatory cytokines with pro-tumorigenic roles or the inhibition of cytokines involved in immune surveillance, altering the composition of the immune cells in the tumor microenvironment (177, 185). Specifically, Rezaeifard et al. suggested that CSCs could alter the cytokines in the tumor microenvironment by demonstrating that the balance between suppressive regulatory T cells (Treg) and T helper cells producing IL17 (Th17) could be affected (186). Chaudhry et al. disclosed further that STAT3 is fundamental for the inhibition of Treg cells development and Th17 differentiation (187). In fact, STAT3 in collaboration with NF κ B, regulates the expression of these genes encoding critical cancer-promoting inflammatory mediators, establishing a crosstalk between cancer and immune cells of the microenvironment and perpetuating the effects of STAT3 activation in cancer cells (188, 189). However, despite the growing evidences showing the interaction of gastric CSCs with the tumor microenvironment, the specific molecules involved and their signaling pathways still need further investigation in order to give rise to safe therapies.

3.2.3. Targeting Therapies

In some cases of gastric cancer, after an intended efficacious treatment, the cancer reappears locally or in distant metastasis. It is believed this could result from the presence of CSCs that were able to resist the therapy applied, revealing that the existence of CSCs is one of the biggest roadblocks in cancer treatment (190). Thus, direct targeting of CSCs could be the key for tumor complete elimination. The therapy against CSCs using specific molecules should eradicate these cells while the conventional therapy eliminates the non-CSCs present in the tumor bulk (Figure 6). Nevertheless, this type of treatment should be administrated in combination with the traditional therapy due to the possibility of cellular plasticity that facilitates the appearance of *de novo* CSCs from non-CSCs (191). Moreover, targeting specific TFs or signaling pathways responsible for maintaining the CSC phenotype could become novel therapies against gastric cancer. For that reason, several clinical trials are being undertaken to explore the efficacy of diverse compounds, that are capable of modulating or inactivating proteins that gastric CSCs use to grow and survive, allowing their elimination (83). Additionally, it is worth to mention that several studies have been conducted to evaluate the efficacy of small molecules in targeting CSCs *in vitro* and *in vivo*, compounds capable of eliminating or reducing the CSC population that could also be part of the path in cancer therapy targeting CSCs (192-196). In fact, in addition to the known chemotherapeutic drugs, several non-oncology compounds have been considered for repurposing in cancer treatment, especially to target CSCs. An interesting class of compounds are the polyether ionophore antibiotics, which include, for example, salinomycin, narasin and monensin (197). Ionophores are lipid-soluble molecules that monocovalently bind to ions and transport them across the lipid bilayer of the cell membrane (197, 198). Therefore, ionophores have a significant impact on maintaining cellular homeostasis and cell survival. Salinomycin (a K⁺ polyether ionophore antibiotic), narisin (a derivative of salinomycin with an additional methyl group) and monensin (a Na⁺ polyether ionophore antibiotic) have been shown to have antitumor effects (197). In particular, salinomycin has been implicated as a potential agent to attack CSCs (199). Zhi et al. showed that gastric cancer stem-like cells were more sensitive to salinomycin compared to gastric non-CSCs (100).

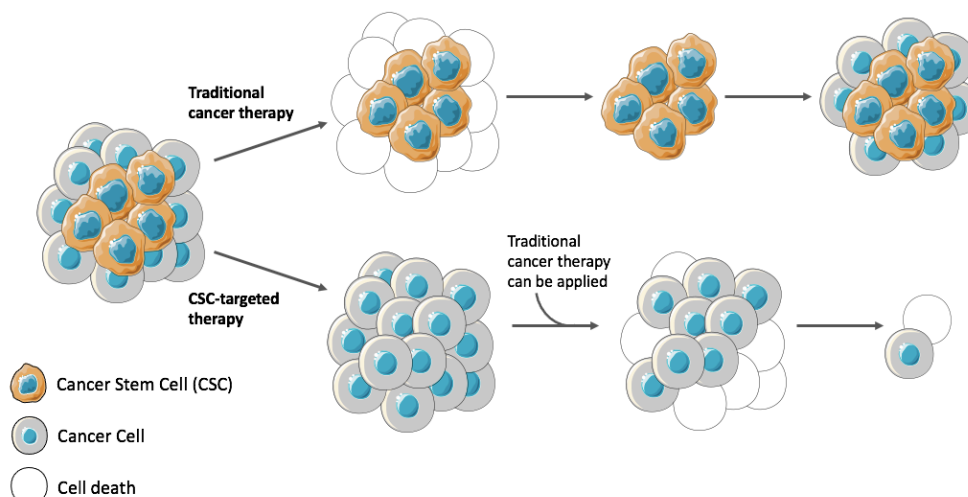


Figure 6 - Schematic representation of the cancer stem cell (CSC) model of drug resistance. Traditional therapy destroys most cancer cells, but the CSCs remain viable, and the tumor recurs and grows again. CSC Targeted therapy eradicates CSCs, and the tumor can no longer grow. And if combined with traditional therapy, the tumor will disappear faster. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

4. CELLULAR PLASTICITY AND CELLULAR REPROGRAMMING

Cellular plasticity refers to the ability of cells to change their phenotypes without genetic mutations in response to environmental stimuli. Recently, Hanahan proposed cellular plasticity as a distinct hallmark capability that can be unlocked during cancer (200). Thus, cancer cells are able to rapidly adapt to tumor environment alterations. Cellular plasticity promotes cancer progression, heterogeneity, and resistance to therapy (201). CSCs also display cellular plasticity. They have the ability to switch between CSC and non-CSC phenotype when necessary (74, 191). The activation of TFs and/or epigenetic modifications have been shown to mediate this interconversion (202). Specially, studies have implied the importance of OCT3/4, SOX2, NANOG and KLF4 in modulating the generation of CSCs and regulation of cellular plasticity (203). Additionally, epigenetic regulation, such as bivalent chromatin state, DNA methylation and histone modifications have also been implicated in CSC plasticity (204). Cellular plasticity plays a relevant role in the ability of CSCs to resist therapy. It has been demonstrated that conventional cancer therapy can directly induce the *de novo* generation of CSC-like cells from non-CSCs, leading to the acquisition of resistance to therapy (205). Metabolic plasticity also plays a significant role in the generation, maintenance, and characteristics of CSCs. These cells can alter their

metabolism to adapt to the different conditions that the microenvironment may be subjected to (206).

Typically, cellular reprogramming refers to the process of reverting specialized cells into iPS cells by rewiring the transcriptional and epigenetic network. During cellular reprogramming, cellular behavior, DNA methylation, histone modifications and gene expression profiles can undergo several modifications (207, 208). Restoring pluripotency by easily inducing cellular reprogramming would be a major advance in several areas, especially in regenerative medicine. In cancer, cellular reprogramming is an interesting approach to convert malignant cells into benign cells, but also to reprogram cancer cells into CSCs in order to generate a larger population of CSCs for experimental manipulation and evaluation of their biological properties, aiming to improve cancer diagnosis and treatment (209, 210). Reprogrammed CSCs appear to exhibit characteristics and genetic profiles comparable to CSCs, serving as a useful platform to investigate the origin and molecular functions of CSCs (211). However, as with somatic cell reprogramming, the efficiency of cancer cell reprogramming is still low due to genetic and epigenetic barriers (122).

It is important to highlight that cellular reprogramming can be carried out both towards differentiation or stemness. In cancer, cellular reprogramming tends to be in the direction of differentiation, that is, in a way, cellular reprogramming attempts to disarm cancer cells from their malignant characteristics, facilitating their elimination through therapy. However, studies in the direction of stemness may contribute to new mechanisms to combat cancer, providing information about the molecular networks essential for malignant characteristics.

4.1. Transcription Factors in Cellular Reprogramming

TFs are proteins that bind to DNA and regulate gene transcription depending on different metabolic stimuli or microenvironmental conditions. Thus, they can stimulate or repress genetic expression (212). TF-mediated cellular reprogramming has diverse applications, including the study of the network of TF involved in cells phenotype and drug discovery (213, 214). Takahashi and Yamanaka described, for the first time, that mouse and human somatic cells can be reprogrammed into iPS cells by the ectopic expression of a combination of four TFs (SOX2, OCT3/4, C-MYC, and KLF4) (122, 215). Later, Shah et al. reported that HMGA1 enhances cellular reprogramming from a somatic cell to a pluripotent stem cell. Their study also showed that cellular reprogramming of somatic cells to iPSCs by using SOX2, OCT4, C-MYC, and KLF4 is enhanced by HMGA1 (216). It is also known that some TFs can be re-expressed or reactivated in cancer cells, playing a crucial

role in the reprogramming of these cells. Cellular transduction of human colon cancer cells with OCT3/4, NANOG and KLF4 promoted enhanced CSC characteristics (217). Suva et al. reported that the combination of POU3F2, SOX2, SALL2, and OLIG2 was sufficient to reprogram differentiated glioblastoma cells into CSCs (218).

4.2. Metabolic Cellular Reprogramming

The role of metabolism in cancer has been widely explored. Recent genomic and proteomic research has provided insights into the molecular mechanisms of cancer metabolism. Reprogramming cellular metabolism is considered as one of the core hallmarks of cancer and plays a fundamental role in regulating gene transcription and repairing DNA damage (219). Knowledge of the process through which cancer cells produce energy to survive and proliferate has proven useful in the discovery of new cancer biomarkers and therapeutic targets (220). In terms of energy production, Otto Warburg observed that cancer cells generally undergo glycolysis rather than oxidative phosphorylation (OXPHOS). This metabolic phenomenon is called aerobic glycolysis or “Warburg effect” (221). Glycolysis is a relatively inefficient way of producing ATP compared to OXPHOS. It converts glucose into pyruvate through a series of metabolic reactions, in an oxygen-independent manner (222). On the other hand, OXPHOS produces most of cellular energy in the form of ATP. It is a fundamental mitochondrial process, where all the energy produced during the oxidation of carbohydrates, lipids and amino acids, and tricarboxylic acid (TCA) is used for the synthesis of ATP (223). Although glycolysis produces less ATP compared to OXPHOS, ATP production in glycolysis is faster than in OXPHOS, which is advantageous for the energy demands of cancer cells (223). Regarding CSCs, there is still no consensus on their metabolic characteristics. Several studies indicate that CSCs are mainly glycolytic, but others point to mitochondrial metabolism as their main source of energy. CSCs also appear to have the ability to adapt their metabolism to microenvironmental changes, opportunely switching energy production from one pathway to another or acquiring an intermediate metabolic phenotype (224). Metabolic reprogramming can happen in different situations, but it usually occurs when there is some stress in the microenvironment. In response to stress, CSCs use their metabolic flexibility to adapt to new microenvironmental conditions in order to survive and maintain stem-like characteristics (225). For example, in a hypoxic microenvironment, gastric CSCs reprogram their metabolism to adapt to lower oxygen levels, upregulating the expression of HIFs that regulate the expression of genes involved in glucose uptake and the glycolytic pathway (226, 227). In fact, HIF-1 α modulates cellular reprogramming through an early glycolytic shift (228). Furthermore, Cao et al. showed that

HMGA1 and C-MYC expression was associated with the glycolysis gene signature in gastric cancer (157).

On the other hand, metabolic reprogramming also enables the generation of iPS cells, thus it is possible that the same occurs in the process of generation of CSCs from non-CSCs. Therefore, iPS cell biogenesis enhanced by metabolic reprogramming may unravel the molecular and cellular mechanisms underlying the *de novo* generation of CSCs. Furthermore, stimulation of aerobic glycolysis can promote the conversion of somatic cells into pluripotent cells (229). In cancer, cellular plasticity and cellular reprogramming are intricately linked, because not all non-CSCs possess the necessary cellular plasticity to allow their reprogramming to the CSC phenotype. Possession of the ability to adapt metabolism to glycolysis allows the transformation of non-CSCs into CSCs (229). Thus, promoting a glycolytic phenotype in non-CSCs through environmental cues may convert these cells, at least those that possess metabolic flexibility, into CSCs.

4.3. Drugs and Small-Molecule-Mediated Cellular Reprogramming

Research has been done to improve the efficiency and safety of cell reprogramming. As TF-mediated cellular reprogramming has low efficiency and also presents risks due to the use of oncogenes and exogenous factors, the use of chemical compounds in cellular reprogramming has emerged as a potential alternative tool to the use of TFs. Drugs and small-molecules can stimulate cellular reprogramming in combination with TFs or by replacing them (230, 231). Most studies search for compounds capable of replacing the main TFs involved in stemness characteristics (e.g., SOX2, OCT4, C-MYC, KLF4, among others). Hou et al. divulged that by using a cocktail of small molecules it is possible to originate pluripotent stem cells from mouse somatic cells (232). They also searched for small-molecules capable of replacing OCT4, for which they transduced the cells with a reported OCT4-GFP reporter system, and the GFP-negative (OCT4-negative) cells were used in a high-throughput screen of more than 10,000 small-molecules molecules. They found that forskolin, 2-methyl-5-hydroxytryptamine and D4476 were able to activate the reporter system in some cells by replacing OCT4 (232). Huangfu et al., also using an OCT4-GFP reporter system, found that the use of valproic acid and a histone deacetylase inhibitor significantly improved the efficiency of cellular reprogramming of somatic cells into iPS cells. Additionally, valproic acid also promotes the induction of iPS cells in the absence of C-MYC expression (233). Ichida et al., by performing a high-content chemical screening, discovered that the small-molecule RepSox (a Tgf- β type I receptor kinase (ALK5) signaling inhibitor)

can replace SOX2 in cellular reprogramming of adult cells into iPS cells, acting through the induction of NANOG (234).

Drugs and small-molecules have been tested to target cancer cells; however, some compounds may promote the induction of a cancer stem-like phenotype. Particularly, chemotherapy has been indicated as a conceivable way to generate CSCs (235). Lu et al. reported that treatment of breast cancer cells with paclitaxel, gemcitabine or carboplatin induced the expression of HIF-1 α . Furthermore, they observed that paclitaxel treatment promoted breast CSCs enrichment by inducing the expression of SOX2 and NANOG in MDA-MB-231 breast cancer cells and the expression of OCT4 and NANOG in SUM-149 breast cancer cells (236). Recently, Lu and Zhang exposed that radiochemotherapy plays a role in the generation of gastric CSCs. They showed that cisplatin and doxorubicin can reprogram about 20% to 27% gastric non-CSCs into cancer stem-like cells through EID3-NAMPT-Wnt/ β -catenin axis in response to DNA damage (237). Gastric CSCs generated by cisplatin or doxorubicin treatment showed an enrichment in the expression of several stemness markers, including SOX2, OCT4, C-MYC, KLF4, NANOG ALDH1 and Lgr5, when compared to gastric non-CSCs (237).

4.4. Senescence-Associated Cellular Reprogramming

Cellular senescence has been described as a permanent and irreversible cell cycle arrest that occurs in response to endogenous and exogenous stresses such as telomere shortening, mitogenic signals, oxidative and genotoxic stress, epigenetic changes, chromatin disorganization, mitochondrial dysfunction, inflammation, radiation, chemotherapeutic agents, nutrient deprivation, DNA damage, oxidative stress or oncogenic activation (238, 239). Senescent cells present alterations in metabolic activity and have the ability to resist apoptosis (239). They also have a distinct secretory phenotype denominated senescence-associated secretory phenotype (SASP). Senescent cells secrete to the microenvironment and neighboring cells numerous soluble factors including cytokines, chemokines, growth factors, and proteases (240). Cellular senescence has been linked with aging, cancer, embryonic development, tissue regeneration and cellular reprogramming (241). In cancer, cellular senescence can suppress tumor growth, but it also has the opposite effect, promoting tumor development through changes in the tumor microenvironment promoted by SASP (242). Additionally, several studies have implicated that treatment with some chemotherapeutic drugs can stimulate a therapy-induced senescence phenotype in cancer cells (243). Several drugs have been implicated in the promotion of a therapy-induced senescence phenotype including doxorubicin, cisplatin,

oxaliplatin, carboplatin, gemcitabine, 5-FU, paclitaxel, docetaxel and irinotecan. All of these drugs provoke several hallmarks of senescence in cancer cells, including prolonged growth arrest, enhanced expression of senescence associated- β -galactosidase (SA- β -gal) and polyploidy (243). This therapy-induced senescence phenotype challenges the notion that cellular senescence is irreversible. Several studies indicate that senescent cells are not permanently arrested and can, in fact, resume proliferation and generate tumors (244). It has been suggested that in response to stress caused by cancer therapy, CSCs may undergo cellular senescence to avoid cytotoxic effects (245). Furthermore, studies have also reported that cellular senescence can promote the reprogramming of non-CSCs toward a cancer stem-like phenotype (246). However, it is not yet fully understood whether the cells that become CSCs are the cells that enter this state of therapy-induced senescence and/or whether they are the cells neighboring the cells that enter this state through SASP-mediated reprogramming.

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RATIONAL & AIMS

We hypothesized that CSCs could be reprogrammed from cancer cells by a limited set of TFs, whose expression can impose CSC properties to cancer cells. The literature and our data, in gastric cancer, suggest that SOX2 is one of the TFs involved in CSC reprogramming and we will use it as a starting point to identify other TFs and major signaling networks associated with CSCs.

Our main goal was to identify the transcriptional regulatory network in gastric CSCs, to harness it for therapeutic strategies and biomarker identification. So, to accomplish that, we defined three specific aims:

- To identify the TFs associated with SOX2 in gastric cancer;
- To reconstruct a gastric CSC using a minimal combination of TFs to find the vulnerabilities of gastric CSCs;
- To evaluate the clinical value of the TFs previously used to obtain the CSC phenotype.

CHAPTER II

A SOX2 REPORTER SYSTEM IDENTIFIES GASTRIC CANCER STEM-LIKE CELLS SENSITIVE TO MONENSIN

This chapter contains the following publication:

Pádua, D., Barros, R., Amaral, A. L., Mesquita, P., Freire, A. F., Sousa, M., Maia, A. F., Caiado, I., Fernandes, H., Pombinho, A., Pereira, C. F., & Almeida, R. (2020). *A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin*. *Cancers*, 12(2), 495.

<https://doi.org/10.3390/cancers12020495>

As mentioned in Chapter I, the identification of gastric CSCs has been mainly performed using cell-surface markers or intracellular enzymes. However, identification with these markers is not specific and presents variations even within the same type of cancer, which makes reproducibility difficult. Therefore, the best CSC markers are those essential for maintaining cellular stem-like characteristics. Among these markers, TFs stand out, including SOX2 and OCT4, which play fundamental roles in promoting stemness and repressing differentiation. Although the expression of SOX2 in gastric cancer divides opinions, its role in this pathology needs to be further explored and its association with gastric CSCs also needs to be clarified. Thus, since many gastric cancer cell lines express SOX2, and also taking into account the success of the SORE6-GFP reporter system developed by Tang et al. in identifying breast CSCs enriched in SOX2/OCT4, we decided to use this system to isolate and characterize gastric CSCs enriched in SOX2 expression. Furthermore, since one of the main characteristics of CSCs is the resistance to therapy, we also took the opportunity to explore, through high-throughput screening of small molecules, compounds capable of selectively eliminating gastric CSCs.

In this Chapter, *A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin*, we show that, by using the SORE6-GFP lentiviral reporter system, we were able to isolate and characterize two cell subpopulations, SORE6+ (CSCs) and SORE6- (non-CSCs), from two human gastric cancer cell lines, AGS and Kato III. SORE6+ cells expressed significant levels of SOX2, whereas SORE6- cells showed no or low expression of SOX2. Furthermore, SORE6+ cells exhibited resistance to 5-FU, improved proliferation and gastrosphere-forming capacity, and increased tumor-forming capacity *in vivo*. Unlike SORE6- cells, SORE6+ cells also exhibited asymmetric division, giving rise to GFP+ and GFP- cells (recapitulating the original cell lineage population) and increased C-MYC, NOTCH1, BAX, CLPTM1L and CYP3A5 levels, compared to SORE6- cells. Furthermore, we found a positive correlation between SOX2 expression and the characteristics of CSCs displayed by SORE6+ cells. Upregulation of SOX2 in SORE6- cells made the cells more proliferative and more resistant to 5-FU, while silencing SOX2 in SORE6+ cells had the opposite effect. To discover a drug that can specifically target gastric CSCs, we performed a high-throughput screening of 1200 small molecules from the Prestwick chemical library. We were able to identify a compound that selectively kills SORE6+ cells. Monensin efficiently decreased the viability of SORE6+ cells while preferentially targeting GFP+ cells in this population.



Article

A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin

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Received: 9 January 2020; Accepted: 15 February 2020; Published: 20 February 2020



Abstract: Gastric cancer remains a serious health burden with few therapeutic options. Therefore, the recognition of cancer stem cells (CSCs) as seeds of the tumorigenic process makes them a prime therapeutic target. Knowing that the transcription factors SOX2 and OCT4 promote stemness, our approach was to isolate stem-like cells in human gastric cancer cell lines using a traceable reporter system based on SOX2/OCT4 activity (SORE6-GFP). Cells transduced with the SORE6-GFP reporter system were sorted into SORE6+ and SORE6− cell populations, and their biological behavior characterized. SORE6+ cells were enriched for SOX2 and exhibited CSC features, including a greater ability to proliferate and form gastrospheres in non-adherent conditions, a larger in vivo tumor initiating capability, and increased resistance to 5-fluorouracil (5-FU) treatment. The overexpression and knockdown of SOX2 revealed a crucial role of SOX2 in cell proliferation and drug resistance. By combining the reporter system with a high-throughput screening of pharmacologically active small molecules we identified monensin, an ionophore antibiotic, displaying selective toxicity to SORE6+ cells. The ability of SORE6-GFP reporter system to recognize cancer stem-like cells facilitates our understanding of gastric CSC biology and serves as a platform for the identification of powerful therapeutics for targeting gastric CSCs.

Keywords: cancer stem cells; gastric cancer; SOX2; monensin; SORE6-GFP reporter system; drug resistance; high-throughput screening

1. Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer mortality worldwide [1–3]. It is a highly heterogeneous disease displaying various histopathological appearances

and molecular signatures. The TNM classification remains the major prognostic marker used to inform treatment decisions in the clinic [4–6]. However, GC is highly heterogeneous even within the same TNM stage, and patient prognosis remains poor.

Cancer stem cells (CSCs) are cells that are capable of initiating and sustaining tumorigenesis and give rise to the phenotypically diverse and more differentiated progeny that composes the bulk of the tumor [7–9]. CSCs are thought to play a major role in driving disease recurrence, due to the increased therapeutic resistance caused by multiple molecular mechanisms, namely high expression of multidrug transporters, enhanced DNA repair, and altered cell-cycle kinetics [10–13]. Thus, understanding CSC biology will be crucial to the development of more effective cancer therapies.

Evidence of CSC existence has been found in a variety of malignancies, including GC [14–19]. Gastric CSCs have been identified in different studies with cell surface molecules namely CD44, CD133, ALDH1, and CD49f [19–22]. GC cells expressing these markers retain sphere-forming and tumor-initiating activities and some of them, namely CD44 and CD133, also have clinical relevance as markers of poor prognosis [23,24]. Thus, in GC, the identification of CSCs has been largely dependent on the combination of distinct cell surface markers that often differ among tumor types, and whose robustness in distinguishing tumorigenic from non-tumorigenic cells has been increasingly challenged [25–27]. Alternatively, transcription factors involved in the establishment of stemness features such as SOX2 and OCT4 could constitute more robust CSC markers [28].

The transcription factor SOX2 belongs to the group B of SOX proteins and plays an important role in cell fate determination, pluripotency, and cell differentiation during development in induced pluripotent stem cells (iPSCs) and in adult stem cells in multiple tissues [29–33]. Hence, SOX2 is unanimously recognized as a key pluripotency factor in multiple tissues and developmental stages. Importantly, in cancer, SOX2 has been increasingly associated with a CSC phenotype in tumors from different tissues, namely of the ovary, prostate, lung, skin, and central nervous system [34–37]. At the clinical level, SOX2 has been shown to act as an oncogene in several epithelial cancers and is a robust marker of poor prognosis [34]. In GC the role of SOX2 is less clear. We and others have shown that SOX2 associates with aggressive features, poor patient outcome, and worse response to chemotherapy [38–41]. However, other studies have shown the contrary, indicating that the relevance of SOX2 in gastric cancer needs further clarification, namely its association with the CSC phenotype [41,42].

OCT4 belongs to the family of POU-domain transcription factors and has a key role in pluripotency and somatic cell reprogramming [43]. Absence of or low OCT4 expression can lead murine embryonic stem cells to differentiate towards trophoblasts, while high expression of OCT4 can promote the differentiation of murine embryonic stem cells into primitive endodermal and mesodermal lineages. Additionally, neural differentiation of embryonic stem cells is enhanced by OCT4 overexpression [44,45]. The role of OCT4 in human cancers is much less studied than SOX2. It has been suggested as a CSC marker in some tumors such as gliomas, and squamous cell carcinomas of the lip and tongue, but the expression and relevance in GC has not been clarified [46–49].

In 2015, Tang et al. generated the novel SOX2-OCT4 reporter system designated the SOX2/OCT4 response element (SORE6-GFP) which they used to isolate breast cancer stem cells with self-renewal and tumor-initiating ability as well as drug resistance, paving the way to use this reporter system to identify gastric CSCs [50].

Thus, the main aims of this work were to isolate and characterize gastric CSCs from gastric carcinoma cell lines using the traceable reporter system based on SOX2/OCT4 activity. We further assessed the role of SOX2 in gastric CSC properties and searched for new drugs with increased activity against these cells.

2. Results

2.1. Isolation of a Small Cellular Subpopulation from Gastric Cancer Cell Lines Enriched in SOX2

AGS and Kato III cells transduced with SORE6-GFP were sorted into two subpopulations, SORE6+ and SORE6− cell populations, according to GFP expression. After cell sorting, in vitro and in vivo assays were used to assess CSC properties. An expression screening was performed to explore the mechanism behind the cellular response to chemotherapy and a high-throughput screening of pharmacologically active small molecules was done in order to identify drugs that can affect gastric CSCs (Figure 1).

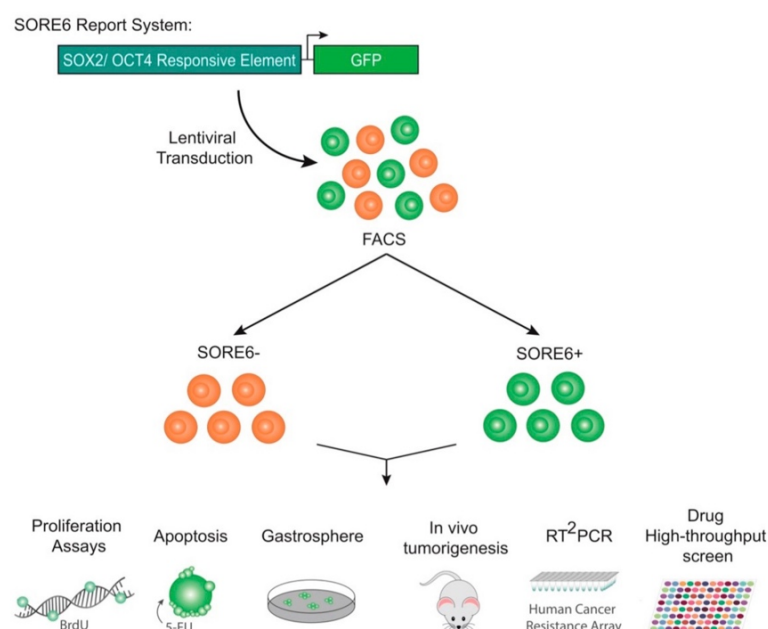


Figure 1. Scheme of overall approach showing the assays performed after the isolation of SORE6+ and SORE6− populations from gastric cancer cells transduced with the SORE6-GFP reporter system.

We first showed that two human GC cell lines (AGS and Kato III) heterogeneously express SOX2. Both cell lines also express OCT4, although at low levels as assessed by qRT-PCR, and undetectable at the protein level by Western blot (Figure 2a). For this reason, we focused the study on SOX2. To characterize the properties of GC cells expressing SOX2, we used the SORE6-GFP reporter system, developed by Tang and his collaborators [50]. This reporter system enables the identification of cellular subpopulations enriched and depleted of these transcription factors. AGS and Kato III cells were transduced with the SORE6-GFP lentiviral system and selected with puromycin to ensure the presence of the reporter construct in all cells. Quantification of the cells that expressed GFP showed that they varied from 8.9 to 10.5% in AGS and from 8.0 to 16.6% in Kato III (Figure 2b). SORE6+ and SORE6− subpopulations from both cell lines were recovered by fluorescence-activated cell sorting (FACS) (Figure 2c) and analyzed for expression of the stem cell transcription factor SOX2 when reached approximately 80% confluence. SORE6+ cells showed an enrichment in SOX2 expression when compared with SORE6− cells and with the respective parental (wild-type, wt) cell lines (Figure 2d). In Kato III, SORE6− cells still expressed some level of SOX2, although lower than SORE6+ and the wt cell line. These results showed that the SORE6-GFP reporter system was able to identify a sub-population of cells, in GC cell lines, that was enriched in the stem cell transcription factor SOX2. Next, we evaluated the expression of cell-surface markers that have been previously identified as CSC markers in GC cell lines, namely ALDH1, CD44, CD133, and CD49f. We only observed an enrichment of ALDH1 in Kato III SORE6+ cells compared with SORE6− cells; however this was not the case in AGS cells where

ALDH1 was not expressed. Interestingly, none of the other markers were enriched in the SORE6+ subpopulation from both cell lines (Figure 2e), supporting the idea that stemness transcription factors are better markers of CSCs. We further observed increased expression of the SOX2 targets, c-MYC and NOTCH1, in SORE6+ cells (Figure S1a), as previously described [51].

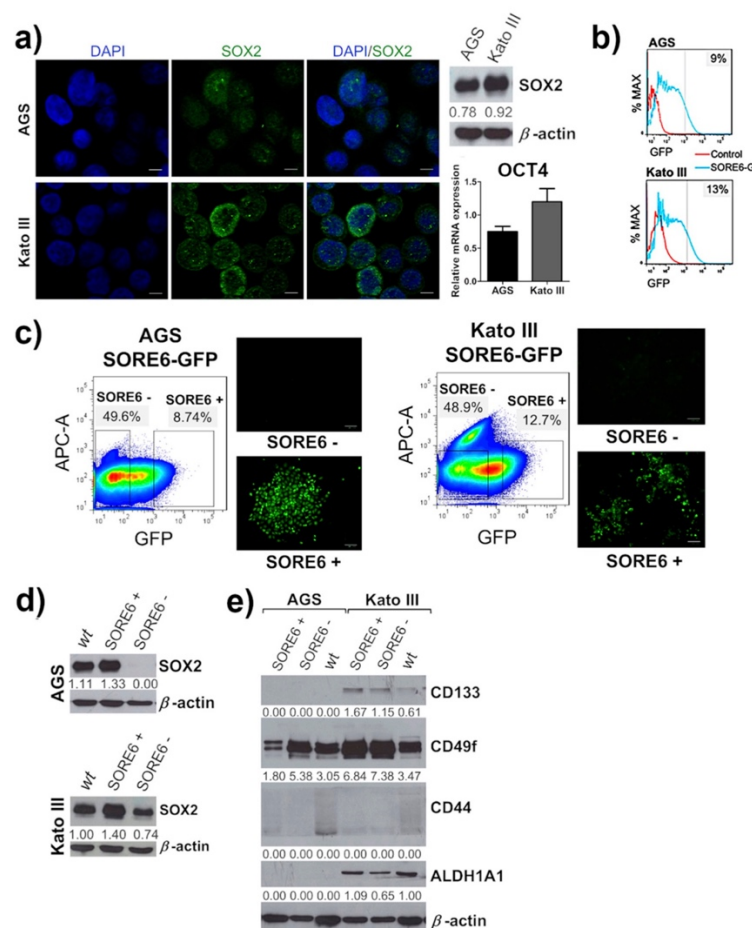


Figure 2. SORE6+ cells have a higher expression of SOX2 than SORE6- cells. (a) Western blot and qRT-PCR analysis exhibiting the expression of SOX2 and OCT4, respectively, in AGS and Kato III cell lines. The representative immunofluorescence images reveal the differences of SOX2 expression per cell in both cell lines. (b) Fluorescence-activated cell sorting (FACS) analysis of AGS and Kato III 10 days after transduction with SORE6-GFP reporter. (c) FACS analysis with gate selection and cell sorting of two cell subpopulations, SORE6+ cells (with GFP expression) and SORE6- cells (without GFP expression), in both SORE6-GFP cell lines, followed by fluorescence images of the respective populations sorted. Scale bar = 100 μ m. APC-A corresponds to an empty channel used to exclude autofluorescent cells from the analysis. (d) SOX2 expression evaluated by Western blot in the FACS-sorted SORE6+ and SORE6- cells from both cell lines. (e) Western blot analysis of the putative gastric cancer stem cell markers ALDH1A1, CD44, CD49f, and CD133 on sorted AGS SORE6 and Kato III SORE6 cell populations. In Western blot β -actin was used as an internal control. Numbers below every Western blot picture represent a semi-quantitative analysis of each line (intensity ratio: “gene of interest”/ β -actin). Pictures show cropped areas of Western blots, the whole images are included in the Supplementary Materials. The qRT-PCR results were normalized to 18S expression. wt corresponds to the parental cell line (AGS or Kato III). Results are mean $\pm$ SD of at least three independent experiments.

2.2. SORE6+ Cell Population Recapitulates the Heterogeneous Cell Line

We then sought to compare the behavior of SORE6+ and SORE6− cellular subpopulations regarding CSC features. SORE6+ and SORE6− cells were kept in puromycin-containing medium after cell sorting to ensure the presence of the reporter construct. In both cell lines, GFP expression decreased with time in the SORE6+ subpopulation, whereas SORE6− cells could not give rise to GFP+ cells up to 10 weeks after sorting (Figure S1b). AGS SORE6+ and SORE6− cell subpopulations were followed by time-lapse microscopy to monitor the emergence of cell heterogeneity. We observed the emergence of SORE6− cells in the SORE6+ subpopulation, reflecting the re-establishment of the heterogeneous population regarding SOX2 expression.

2.3. The SORE6+ Cells Were Highly Proliferative and Enriched in Tumor-Initiating Cells

We evaluated the proliferation of both sorted cellular subpopulations from AGS and Kato III through bromodeoxyuridine (BrdU) incorporation. SORE6+ cells had a significantly higher rate of proliferation when compared with the SORE6− cells and the corresponding wt cell lines (Figure 3a and Figure S2b). A key property of CSCs is the ability to form sphere-like structures under non-adherent conditions and serum-free medium supplemented with growth factors to minimize the influence of external signals. In these conditions, the SORE6+ subpopulations from both cell lines were enriched in cells with increased ability to form gastrospheres, presenting a significantly higher number of gastrospheres larger than 100 µm in comparison to wt and SORE6− cells (Figure 3b). Then, we evaluated the tumor-initiating ability of SORE6+ and SORE6− cells after subcutaneous inoculation in athymic nude mice. The SORE6+ subpopulations were enriched in cells with improved tumor-initiating ability, since from 13 mice inoculated with AGS SORE6+ cells, 12 developed tumors and all mice inoculated with KATO III SORE6+ formed tumors. In contrast, only seven out of 13 mice inoculated with AGS SORE6− cells generated tumors and half of the 10 mice inoculated with Kato III SORE6− had a tumor (Figure 3c and Figure S2c).

Tumors obtained from SORE6+ cells expressed SOX2 abundantly and some expression of SOX2 was also observed in tumors obtained from the SORE6− subpopulations. This was expected in KATOIII cell line, as SOX2 was never completely absent in the SORE6− cells. In contrast, in AGS SORE6− cells, SOX2 expression was completely absent in vitro but regained to some extent in vivo (Figure S2d), suggesting phenotypic plasticity as previously described by Tang et al. [47].

2.4. The SORE6+ Cells Are More Resistant to 5-Fluorouracil (5-FU) Treatment

CSCs are more resistant to chemotherapeutic drugs, which is a critical property leading to tumor recurrence and significant clinical implications. To assess this property in the subpopulations obtained with the SORE6-GFP reporter system, cells were incubated with 5-FU, which is the standard-of-care in the treatment of GC, and the level of apoptosis was determined [52]. SORE6+ subpopulations from both cell lines were more resistant to 5-FU in comparison to the SORE6− cells and respective wt cell lines. In contrast, SORE6− subpopulations were the most sensitive to the drug. After 48 h of treatment with 5-FU, around 13% of AGS SORE6+ cells and about 55% of Kato III SORE6+ cells were in apoptosis. Conversely, approximately 77% of AGS SORE6− cells and 79% of Kato III SORE6− cells were apoptotic. AGS SORE6+ cells were more resistant to 5-FU than Kato III SORE6+ cells (Figure 4a and Figure S3a). Apoptosis was caspase-dependent in AGS but not in KatoIII (Figure S3a), as previously described [53]. To search for the molecular mechanism involved in drug resistance in SORE6+ cells, we used the RT2 Profiler PCR Array Human Cancer Drug Resistance kit which allowed us to profile the expression of 84 genes involved in cellular responses to chemotherapy (Figure 4b). The screening identified nine genes with a significantly different expression between AGS SORE6+ and AGS SORE6− cells. Of these, three were upregulated in AGS SORE6+ cells: BAX, CLPTM1L, and CYP3A5, and six were downregulated in these cells: CDKN1B, ELK1, ERBB2, IGF1R, SOD1 and RARG (Figure 4c). We next performed qRT-PCR for BAX, CLPTM1L, CYP3A5, CDKN1B, SOD1, and RARG in the two subpopulations from

both cell lines. We obtained the same results in AGS, whereas in KatoIII only the upregulated genes were confirmed (Figure S3b). These results suggest that relevant mechanisms of drug metabolism might be involved in the resistance to 5-FU and indicate also the existence of cell type specific drug resistance mechanisms.

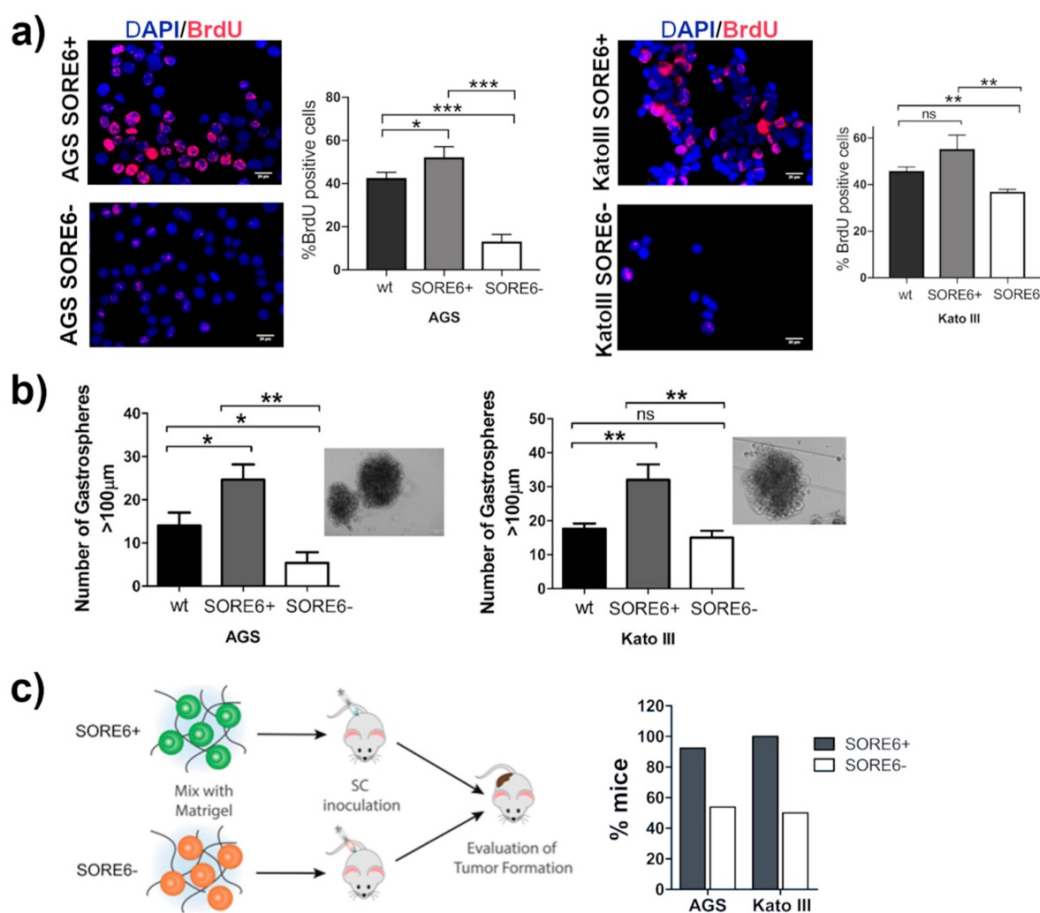


Figure 3. SORE6+ cells have a higher ability to proliferate, to form gastrospheres in non-adherent conditions and a larger in vivo tumor-initiating capability than SORE6- cells. (a) Immunofluorescence displaying the BrdU incorporation in AGS and Kato III SORE6 subpopulations followed by the results obtained by FACS analysis of BrdU incorporation in AGS wt, SORE6+, and SORE6- cells and Kato III wt, SORE6+, and SORE6- cells to evaluate the proliferation ability of each cell line (Figure S2a,b). Scale bar = 100 μ m. (b) Gastrosphere-forming ability of AGS wt, SORE6+, and SORE6- cells and Kato III wt, SORE6+, and SORE6- cells. Only spheres with a diameter >100 μ m were considered as gastrospheres. Representative phase-contrast images of the gastrospheres are shown. Scale bar = 100 μ m. Results are mean $\pm$ SD of three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$). ns: Not significant. (c) Percentage of mice that developed a tumor after subcutaneous inoculation of 5×10^5 AGS SORE6+ or SORE6- cells or with 3×10^5 Kato III SORE6+ or SORE6- cells.

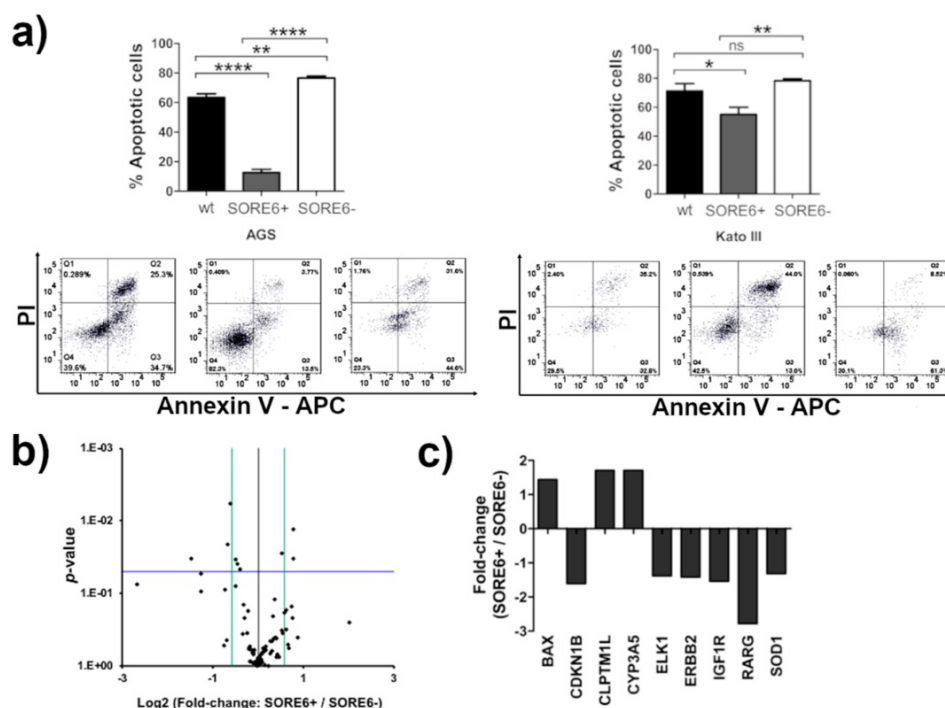


Figure 4. SOX2+ cells are more resistant to 5-fluorouracil (5-FU) than SOX2- cells. (a) Annexin V/ propidium iodide (PI) assay results after FACS analysis of AGS and Kato III wt and SOX2 subpopulation after 48 h treatment with 5-FU. Results are mean $\pm$ SD of three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$, and **** $p \leq 0.0001$), ns-no significant. (b) Gene expression analysis of 84 genes involved in the response to chemotherapy in AGS SOX2+ and SOX2- cells using the RT2 Profiler PCR Array Human Cancer Drug Resistance. Volcano plot of AGS SOX2+ cells in comparison to AGS SOX2- cells ($n = 3$). The horizontal blue line represents the threshold of statistical significance ($p = 0.05$) and the green lines corresponds to the fold change cut-off ≥ 1.5 . (c) Genes that showed a significant fold change, up- or down-regulation, ($p \leq 0.05$) in AGS SOX2+ cells compared to AGS SOX2- cells.

2.5. SOX2 Has a Prominent Role in Determining the CSC Phenotype of SOX2+ Cells

In order to assess the relative role of SOX2 in defining the CSC properties previously observed in SOX2+ and SOX2- populations, we downregulated SOX2 expression in SOX2+ cells from both cell lines. SOX2 knockdown in SOX2+ cells resulted in a significant decrease in cell proliferation compared to the CTRL siRNA and the apoptosis analysis after 48 h treatment with 5-FU revealed that cells with SOX2 downregulation presented a significantly higher sensitivity to the drug in comparison with the CTRL siRNAs. Upon SOX2 knockdown, 49% of the cells were apoptotic, whereas only 21% of the cells treated with the CTRL siRNAs were in apoptosis. In Kato III SOX2+ cells with SOX2 downregulation, 61% of the cells were apoptotic, contrasting with the 38% of apoptotic cells detected in the control (Figure 5a). Overall, these results suggest that SOX2 plays a key role in determining the properties exhibited by SOX2+ cells. To further confirm this, we overexpressed SOX2 in the SOX2- subpopulation from both cell lines. The proliferation results showed that SOX2- cells transiently transfected with SOX2 significantly increased proliferation when compared with the control cells. They also became more resistant to 5-FU, with 44% of the cells overexpressing SOX2 in apoptosis, in contrast to the 76% of cells observed in the control, in the AGS SOX2- subpopulation. In Kato III SOX2- subpopulation, nearly 45% of cells transiently transfected with SOX2 entered apoptosis, whereas in the control, around 67% of cells were in apoptosis (Figure 5b).

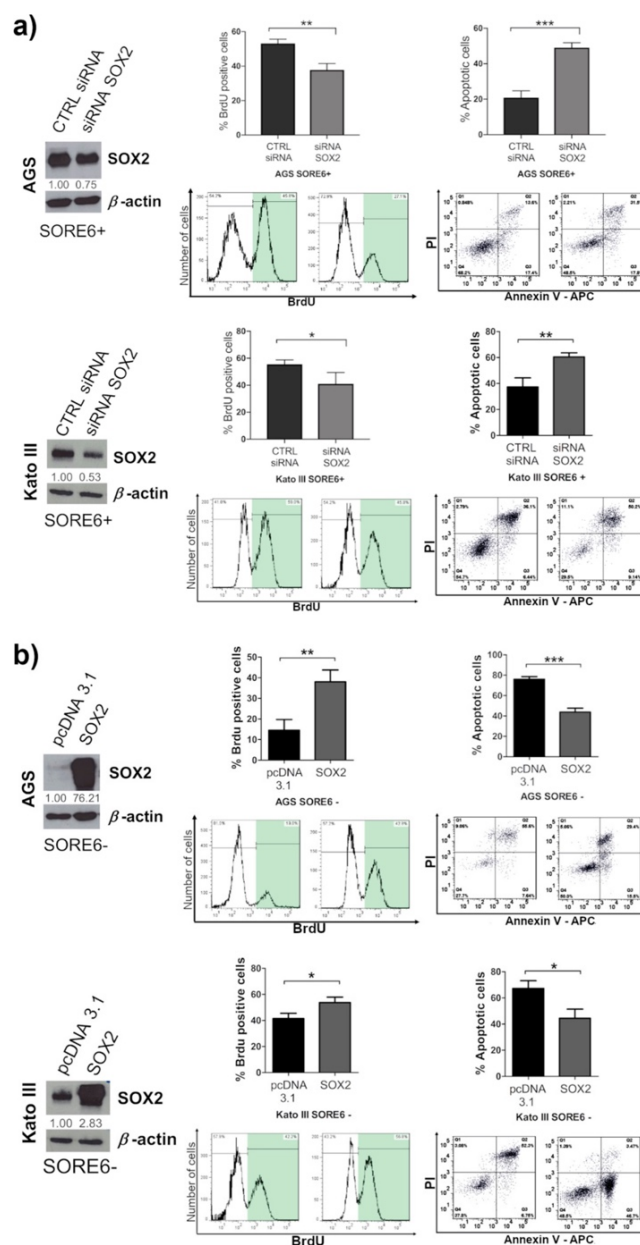


Figure 5. SOX2 has a key role on cell proliferation and on 5-FU resistance. **(a)** Western blot of AGS SORE6+ and Kato III SORE6+ cells 72 h after transient transfection with an siRNA for knockdown of the SOX2 protein (siRNA SOX2); the corresponding control is CTRL siRNA. This is followed by the results of BrdU incorporation for proliferation analysis and annexin V/PI assay results. Cells were used for apoptosis analysis after 48 h incubation with 5-FU. **(b)** Western blot of AGS SORE6- and Kato III SORE6- cells 72 h after transient transfection with the aim of overexpressing the SOX2 protein (SOX2); the corresponding control is pcDNA3.1. This is followed by the results of BrdU incorporation for proliferation analysis and annexin V/PI assay results, used for apoptosis analysis after 48 h of incubation with 5-FU. In Western blot β-actin was used as an internal control. Numbers below every Western blot picture represent semi-quantitative analysis of each line (intensity ratio: SOX2/β-actin). Pictures show cropped areas of Western blots, the whole images are included in the Supplementary Materials. Results are the mean ± SD of three independent experiments. Significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

2.6. Monensin Selectively Kills SORE6+ Cells

In order to identify new drugs that could be more efficient in targeting SORE6+ cells, we screened 1200 compounds from the Prestwick chemical library in both AGS SORE6 subpopulations and evaluated the loss in cell number caused by each one in the SORE6+ and SORE6− subpopulations. Compounds inducing a decrease in the cell number higher than three times the standard deviation (3SD) of the mean of the negative control (DMSO) were considered hits. From these primary hits, 41 compounds displayed a selective effect towards either SORE6+ or SORE6− subpopulations and were picked for testing their activity in a dose–response assay. Six compounds were then selected for validation based on their selective reduction of cell number towards one of the subpopulations and/or on the decrease of GFP+ cells within the SORE6+ population. This stepwise approach allowed us to identify two compounds that impacted preferentially on AGS SORE6+ and four on AGS SORE6− cell number. One of these compounds, monensin, more efficiently decreased the cell number of the AGS SORE6+ cells (Figure 6a) while preferentially targeting the GFP+ cells within this population (Figure 6b). This result was replicated in Kato III SORE6 + and SORE6− cells where, although a statistically different effect on cell number could not be observed (Figure S3c), the GFP+ subpopulation within the Kato III SORE6+ cells was significantly more affected by monensin than the GFP− subpopulation (Figure S3d). The identification of monensin's ability to reduce the number of cells with high GFP levels in the SORE+ population suggests that there is a possibility for CSCs in gastric tumors to be therapeutically targeted.

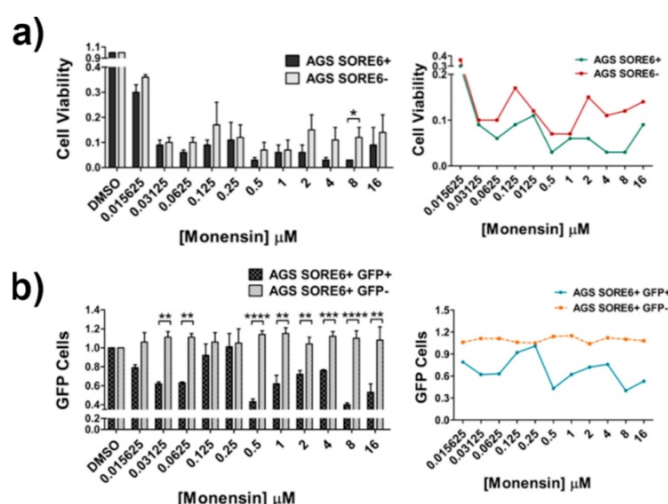


Figure 6. Monensin is more effective in SORE6+ cells. (a) AGS SORE6+ and SORE6− cells viability results after 48 h of treatment with monensin, normalized for the DMSO. (b) Number of GFP+ and GFP− cells in the AGS SORE6+ population after 48 h of treatment with monensin, normalized for the DMSO. Results are mean $\pm$ SD of three independent experiments. Significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$).

3. Discussion

In this study, making use of the SORE6-GFP reporter system, we identified two cellular subpopulations, SORE6+ and SORE6− cells, with differential expression of SOX2 in two GC cell lines. Further, we demonstrated that SORE6+ cells present features of CSCs mostly regulated by SOX2. We further identified differentially expressed genes that could be involved in drug resistance of SORE6+ cells and monensin as a new drug with superior toxicity in gastric CSCs.

The CSC phenotype has been extensively studied and it has become increasingly clear that one of the main challenges is the identification of reliable CSC markers. An essential tool for the identification and isolation of candidate CSCs has been the expression of unique combinations of cell surface markers, which is an approach that has allowed the isolation of CSCs in many solid malignancies using flow

cytometry or magnetic cell sorting. In GC, a number of cell surface markers have been identified as CSC markers (CD44, CD133, ALDH1, and CD49f) [19,21,22,54]. However, several studies revealed that these markers lack specificity and furthermore cannot be used for real-time assessment of the CSC behavior [26,55]. To address this issue, Tang et al. developed a new lentiviral reporter system (SORE6-GFP) based on SOX2/OCT4 stemness marker activity and successfully used it to identify, quantify and isolate a small subpopulation with CSC characteristics in breast cancer cell lines and in the prostate [50,56]. Here, it was used for the first time in GC cell lines and showed that SOX2 is not only a marker but also a driver of the CSC phenotype in GC. SOX2 is an essential component in the cocktail of transcription factors used to generate iPSCs. It is required in embryonic stem cells and it is expressed in adult multipotent stem cells in different tissues such as the stomach, esophagus, lung, skin, and brain [31]. Thus, it is conceivable that it might be a marker and an inducer of the CSC phenotype. Sox2-expressing cells are able to differentiate into all cell types found in a gastric unit, and ablation of the Sox2+ population prevents renewal of the gastric epithelium [31]. SOX2 expression has been associated with multiple tumor types; however, at the clinical level there is a lack of consensus as to whether it is a marker of good or poor prognosis. Still, the vast majority of studies associate SOX2 expression with poor patient prognosis [37,39]. We have previously shown that SOX2 is a marker of poor patient prognosis in GC, which would be in accordance with the more aggressive behavior of the cells that express SOX2, namely increased proliferation, increased tumor-formation, and increased drug resistance, observed in this study [38]. In addition, it is well established that SOX2 regulates cellular proliferation in many cancer types; for instance, its overexpression in the lungs of mice induces rapid proliferation and, in some cases, leads to adenocarcinomas [57]. On the other hand, the knockdown of SOX2 in different cell lines, including gastric, significantly reduces proliferation in vitro and tumor growth [40,58] SOX2 has also been shown to promote tumor invasion, migration, and metastasis in numerous cancers [59]. In cervical squamous cell carcinoma, high expression of SOX2 along with high expression of OCT4 indicated resistance to radiotherapy, and both factors were important predictors of poor patient survival [60]. Our results are in accordance with these studies and with the recent study by Chen et al. that shows that OCT4 and SOX2 overexpression in GC cells induces changes in the biological behavior of the cells towards a CSC phenotype [61]. However, in our study, the reporter system used allowed us to separate cells based on the endogenous expression of these transcription factors, mainly SOX2. Furthermore, we observed concordance between SOX2 and ALDH1A1 expression in Kato III, but not with the other previously identified gastric CSC-surface markers. This might result from the existence of multiple CSC populations or CSC plasticity, which requires further studies [62]. In this study, we highlight the relevance of SOX2 in the behavior of gastric cancer but there are other molecular players, such as the recently identified USF1 whose interaction with the CSC phenotype needs to be addressed [63].

An important clinical readout of the CSC phenotype is the increased drug resistance, namely to 5-FU in GC, which in patients might be related with the worse prognosis, already described [64,65]. We confirmed that SORE6+ cells have increased resistance to apoptosis and screened genes that could be involved in this process. Interestingly we found that SORE6+ cells have increased expression of two genes known to enhance resistance to drugs by different mechanisms. CLPTM1L, also known as cisplatin resistance-related protein 9, confers resistance to apoptosis caused by genotoxic agents and leads to up-regulation of the anti-apoptotic protein, Bcl-xL [66,67]. CYP3A5 encodes a member of the cytochrome P450 superfamily of enzymes, which have multiple functions in drug metabolism and in facilitating the elimination of drugs [68]. Interestingly, SORE6− cells were enriched in, among other entities with a less clear role, a known inhibitor of proliferation (CDKN1B) and an inducer of differentiation (RARG), which could be contributing to the less aggressive phenotype of these cells [69,70]. These results indicate that the mechanisms involved in drug resistance in the SORE6+ cells, are not related with regulation of the cell cycle. These cells are more proliferative, which in principle should render them more sensitive to 5-FU; however, this is not the case. Here, we obtained

clues that multidrug resistance mechanisms are at stake in the SORE6+ cells, in accordance to what has been demonstrated for CSCs in multiple tumors [65,71,72].

The reporter system used in this study proved to be useful for the screening of drugs with impact in CSCs. We identified monensin, a polyether ionophore antibiotic, currently used as an anti-bacterial and anti-parasitic drug, to be toxic to cancer cells with superior activity over CSCs [73]. Ionophores are organic molecules that bind metal cations and form lipid-soluble complexes, facilitating their transport across cellular membranes [74]. Like other ionophore antibiotics, monensin induces apoptosis and causes growth inhibition in diverse types of cancer cells, even in those that display multidrug resistance [75,76]. At the molecular level, monensin has pleiotropic effects affecting multiple cancer-related pathways such as the E2F/DP1, STAT1/2, NF κ B, AP-1, Wnt, and Elk-1/SRF pathways, likely dependent on the cellular model [77]. Interestingly, another structurally related compound, the ionophore salinomycin, was shown to specifically kill human CSCs and inhibit *in vivo* tumorigenesis. Salinomycin induces cell apoptosis, disturbing the equilibrium of Na⁺/K⁺ ions in cellular membranes including mitochondria and cytoplasm, overcoming resistance to drugs by multidrug-resistant genes [78]. In this study, we showed that SORE6+ cells were resistant to 5-FU, but sensitive to monensin, suggesting that monensin is able to bypass the common mechanisms of drug resistance present in CSCs. Being an ionophore, monensin also alters the concentration of cations inside the cells. This has been demonstrated in prostate cancer cell lines, where intracellular Ca⁺ levels were reduced in the presence of the drug and this was the primary mechanism leading to cell cycle arrest and apoptosis [79].

4. Materials and Methods

4.1. Cell Culture

Two human gastric carcinoma cell lines were used in this study, AGS (ATCC, CRL-1739, Manassas, VA, USA) and Kato III (ATCC, HTB-103, Manassas, VA, USA), as well as the human embryonic kidney cell line, HEK293T (kindly provided by Dr. João Relvas from Glial Cell Biology Group, i3S, Porto, Portugal). AGS and Kato III cell lines were cultured in RPMI medium 1640 with 25 mM Hepes and GlutaMAX-1 (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, France). After sorting, the cell lines were cultured in DMEM without phenol red (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA) supplemented with 10% FBS, 1% sodium pyruvate (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA), and 1% penicillin-streptomycin (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA). The HEK293T cell line was cultured in DMEM with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA). All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

4.2. Viral Supernatant Production and Cell Transduction

The mCMVp-dsCopGFP-PURO (Ø) or SORE6-mCMVp-dsCopGFP-PURO (SORE6-GFP) lentiviral reporters were a kind gift from Dr. Lalage M. Wakefield, NCI, USA. The SORE6-GFP is a lentiviral reporter construct in which six concatenated repeats of a composite SOX2/OCT4 response element (SORE6) from the proximal human NANOG promoter were coupled to a minimal cytomegalovirus (CMV) promoter and used to drive expression of GFP [50]. SORE6-GFP and the empty vector were cotransfected with the psPAX2 packaging plasmid and the pVSV-G envelope plasmid into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a 1 µg DNA: 1.5 µL lipofectamine ratio in serum- and antibiotic-free Opti-MEM medium (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA), and cells were incubated for 24 h. After incubation, cells were exposed to fresh HEK293T culture medium and left undisturbed for 48 h. All cell lines were exposed to viral supernatants (Ø or SORE6-GFP) diluted 1:5 in fresh medium for 72 h. Transduced AGS and Kato III cultures were selected with 5 µg/mL of puromycin (Gibco, Life Technologies, Thermo Fisher, Waltham,

MA, USA) for at least 10 days prior to fluorescence-activated cell sorting (FACS), to ensure the presence of SORE6-GFP vector in all cells. GFP expression was monitored with the ZOE Fluorescent Cell Imager (Bio-Rad, Hercules, CA, USA).

4.3. Fluorescence-Activated Cell Sorting (FACS)

Transduced cells were collected by trypsinization, washed with phosphate-buffered saline (PBS), and resuspended in PBS with 5% FBS. AGS and Kato III cells transduced with SORE6-GFP were sorted using a FACS Aria II Cell Sorter flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA), and cells transduced with $\emptyset$ viral supernatant were used as negative controls for gating assessment. Cells with and without GFP signals—SORE6+ and SORE6−, respectively—were seeded in six-well plates at a density of 3.5×10^4 to 7.5×10^4 cells/mL. The data were analyzed using FlowJo software (version 7.6.1).

4.4. Western Blot and Immunofluorescence

To prepare whole cell extracts, cell pellets were washed with PBS and resuspended in cold RIPA lysis buffer (50 mM Tris-HCl pH = 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) supplemented with the complete protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM PMSF, and 1 mM Na_3VO_4 . Then, 30 μg of total protein extracts were separated by standard SDS-PAGE using the Precision Plus Protein Standard Dual Color protein marker (Bio-Rad, Hercules, CA, USA). After separation, proteins were transferred to a nitrocellulose membrane (Amersham, GE Healthcare, Chicago, IL, USA). Membranes were washed with TBS-1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and blocked with either 5% non-fat milk in TBS-1% Tween-20 or 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with the following primary antibodies: SOX2 (diluted 1:500; Cell Marque, Sigma-Aldrich, St. Louis, MO, USA); CD49f (ITGA6) (diluted 1:1000; Sigma-Aldrich, St. Louis, MO, USA); CD133 (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); CD44 (diluted 1:500; Cell Signaling Technology, Danvers, MA, USA); ALDH1A1 (diluted 1:500; Abcam, Cambridge, UK); c-Myc (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); NOTCH1 (diluted 1:500; Santa Cruz Biotechnology, Dallas, TX, USA); PARP (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); cleaved PARP (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); caspase 3 (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); cleaved caspase 3 (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); caspase 7 (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); caspase 9 (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); and β -actin (diluted 1:4000; Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were washed with TBS-1% Tween-20 and incubated for 1 h at room temperature with the respective HRP-conjugated secondary antibody: rabbit IgG (HRP) (diluted 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA); mouse IgG (HRP) (diluted 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), or goat IgG (HRP) (diluted 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA). Signal detection was performed using the ECL detection kit (Amersham, GE Healthcare, Chicago, IL, USA). β -actin was used as loading control. For immunofluorescence analysis, AGS and Kato III cell lines were plated in six-well plates, with five coverslips in each well, at a density of 6×10^4 cells/mL in RPMI 10% FBS and allowed to attach overnight. When cells reached about 70%–80% confluence, medium was removed, cells were washed with PBS (1X), fixed with cold methanol and washed again with PBS (1X). Then, the coverslips were blocked with 1 μL goat normal serum (Dako, Denmark): 5 μL PBS 0.5% Tween-20 0.05% BSA for 30 min in the dark at room temperature. Next, the coverslips were incubated with the primary antibody SOX2 (diluted 1:50) overnight at 4 °C. After incubations, the coverslips were washed twice with PBS (1X) and incubated with the secondary antibody goat anti-rabbit Alexa Fluor 488 (diluted 1:100; A11034, Thermo Fisher, Waltham, MA, USA) for 45 min in the dark at room temperature. 4',6-diamidino-2-phenylindole (DAPI, dilution 1:100; Sigma-Aldrich, St. Louis, MO, USA) was added to the coverslips for nuclei staining. The coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and

pictures were taken using a Carl Zeiss fluorescent microscope (Carl Zeiss Microscopy, Oberkochen, Germany). Uncropped western blots are shown in Figure S4.

4.5. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using the TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. Concentration and quality of the RNA were assessed using the NanoDrop ND-1000 spectrometer (V3.5.2 Software). Total RNA was converted to cDNA for 10 min at 65 °C in a thermocycler (Bio-Rad, Hercules, CA, USA) in a final reaction volume of 20 µL, containing 1 µL of random primers (100 ng/µL), 4 µL of Buffer 5x, 2 µL of DTT, 2 µL of dNTPs (10 mM), 0.3 µL of RNaseOUT (40 U/µL), and 0.5 µL of SuperScript III Reverse Transcriptase (200 U/µL) in DEPC-treated water (all reagents were purchased from Invitrogen, Carlsbad, CA, USA). For the PCR, each reaction was prepared with 4 µL of cDNA diluted 1:20 in DEPC-treated water, 10 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.6 µL of each primer (10 µM), and 4.8 µL of DEPC-treated water (Invitrogen, Carlsbad, CA, USA). The primers used were the following: 18SFor: CGCCGCTAGAGGTGAAATTC; 18SRev: CATTCTTGGCAAATGCTTTCG; OCT4For: TGCAGCAGATCAGCCACAT; OCT4Rev: ACACTGGTCCCCCTGAGAAA; BAXFor: TTTTGCTTCAGGGTTTCATC; BAXRev: GACACTCGCTCAGCTTCTTG; CLPTM1LFor: TCTGATACACAGCAGATCGAGG; CLPTM1LRev: AGTTGTCCGCCATCACGTTC; CYP3A5For: TCGAAGGTCTTTAGGCCCGAG; CYP3A5Rev: GGTGAAGGTTGGAGACAGCA; CDKN1BFor: CCAAAGGTGCCTGCAAGGTG; CDKN1BRev: AGAAGAATCGTCGGTTGCAGGT; RARGFor: CATCCAAGAGACTGCCCGAC; RARGRev: CGTAGCTGCTGGAGTGGG; SOD1For: GCCAAAGGATGAAGAGAGGCAT; SOD1Rev: ACATCGGCCACACCATCTTT. The reactions were performed in a 7500 Fast Real-Time PCR System using the software v2.0.6 (Applied Biosystems, Foster City, CA, USA). Each experiment was carried out in triplicates and three negative controls (without cDNA) were included in each plate. The housekeeping control gene, 18S, was used for normalization of target gene abundance. Data was analyzed by the comparative $2\Delta\Delta CT$ method [80].

4.6. Sulforhodamine B (SRB) Assay

For the 5-fluorouracil (5-FU, Sigma-Aldrich, St. Louis, MO, USA) IC₅₀ determination, a dose–response curve between the drug concentration and the percentage of cell growth inhibition was performed for each cell line. For that, AGS and Kato III cell lines were seeded in 96-well plates at a density of 1×10^4 and 2×10^4 cells/mL respectively and treated for 48 h with a range of concentrations between 6.25 and 100 nmol/mL of 5-FU (Sigma-Aldrich, St. Louis, MO, USA). Cells were fixed with cold 10% (w/v) TCA (Merck Millipore, Burlington, MA, USA), stained with 0.4% (w/v) SRB (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and then washed with 1% (w/v) acetic acid. The dye was solubilized with 10mM Tris Base (pH = 10.5) and the optical densities were measured at 510 nm using the Synergy Microplate Reader (Biotek, Winooski, VT, USA).

4.7. Annexin V/Propidium Iodide (PI) Assay

Cell lines were seeded in six-well plates at a density of 0.6×10^5 cells/mL and were allowed to attach overnight. The following day, cells were treated with the concentration of 5-FU corresponding to the IC₅₀ value determined for AGS and Kato III wild-type cell lines. Cells treated only with dimethyl sulfoxide (DMSO, Applichem) were used as control. After 48 h, cells were collected by trypsinization and pellets were resuspended in Binding Buffer 4x (annexin V Apoptosis detection Kit, eBioscience, Affymetrix, Santa Clara, CA, USA). Each sample was first stained with annexin V Alexa Fluor 594 conjugate (Invitrogen, Carlsbad, CA, USA) or annexin V APC (ImmunoTools, Germany) and then with propidium iodide (PI) (annexin V Apoptosis detection Kit, eBioscience, Affymetrix, Santa Clara, CA, USA). The percentage of apoptosis was measured using the FACS Aria II flow cytometer. The results were analyzed using FlowJo software (version 7.6.1).

4.8. BrdU Incorporation Assay

Cells were cultured in six-well plates at a density of 0.8×10^5 cells/mL and, 24 h later BrdU was incorporated in the cell culture medium at the ratio of 1:1000, for 1 h to 2 h at 37 °C. The cells were harvested by trypsinization and cell pellets fixed in ice-cold methanol (Chem-Lab, Zedelgem, Belgium). The cells were resuspended in HCl 4 M (Mallinckrodt Baker, Avantor, Allentown, PA, USA) for 20 min at room temperature followed by two washing steps with PBS and a blocking step with PBS containing 0.5% Tween 20 and 0.05% BSA for 10 min. The cells were incubated with the primary antibody against BrdU (1:20, monoclonal mouse, Bu20a, Dako, Denmark) for 1 h at room temperature, washed with PBS, and incubated with the secondary antibody labelled with FITC (1:150 μ L, polyclonal rabbit anti-mouse, Dako, Denmark) for 30 min, washed again and resuspended in PBS. The percentage of BrdU positive cells was assessed using the FACS Aria II flow cytometer. The data was analyzed using FlowJo software (version 7.6.1). BrdU incorporation was also observed by immunofluorescence, methodology previously described in 4.4, with an additional step after methanol fixation where the cells were treated with HCl 4 M for 20 min at room temperature. The coverslips were incubated with the primary antibody BrdU (diluted 1:20) overnight at 4 °C. The goat anti-mouse Alexa Fluor 594 (diluted 1:100; A11032, Thermo Fisher, Waltham, MA, USA) was used as secondary antibody.

4.9. Gastrosphere Formation

Cells were trypsinized and disaggregated with a 25-gauge needle (BD Bioscience; Franklin Lakes, NJ, USA) to obtain single-cell suspensions in cold PBS. Cells were then plated at 1×10^3 cells/well in 24-well plates coated with 12 g PolyHEMA (Sigma-Aldrich, St. Louis, MO, USA)/1 L of 95% EtOH. Cells were grown for 10 days in serum-free DMEM medium containing B27 supplement, N2 supplement, 10 ng/mL of bFGF, 20 ng/mL of hEGF, and 1% of penicillin-streptomycin, at 37 °C and 5% CO₂ in a humidified incubator. After 10 days, cells were examined and the number of gastrospheres with more than 100 μ m in diameter were counted in a total of 10 independent fields per well in a Zeiss optical microscope.

4.10. Xenografts in Nude Mice

Animal experiments were approved by the i3S animal ethics committee (CEA–Comissão de Ética Animal) and licensed by the Direção Geral de Alimentação e Veterinária (DGAV), code DAGV 0421/000/000/2015. Experiments were performed in the animal facility of i3S according to the institutional guidelines regulated by Decreto-Lei 113/2013 (Portugal), which is the national transposition of the European Directive 2010/63/EU on the protection of animals used for scientific purposes and FELASA guidelines. Female athymic nude mice (NIH(S)II-nu/nu [81]) aged 6 to 8 weeks were subcutaneously inoculated with 5×10^5 AGS SORE6+ or AGS SORE6– cells or with 3×10^5 Kato III SORE6+ or Kato III SORE6– cells resuspended in 1:1 RPMI medium: Matrigel matrix phenol red-free (BD Bioscience; Franklin Lakes, NJ, USA) using a 25 gauge needle. Animals were observed, and their weight measured once a week. They were euthanized one to two months after inoculation and tumors were resected and fixed in neutral buffered formalin 10%.

4.11. Immunohistochemistry

After fixation, the mice tumors were processed, embedded in paraffin and sectioned with a microtome (Microm HM 335 E) at 3- μ m thickness. Tumor sections were deparaffinized in xylene and rehydrated through a graded series of ethanol (EtOH) concentrations. Antigen retrieval was performed by heat-induced (98 °C) in 1 mL EDTA buffer 10 mM pH = 8.0 (Thermo Fisher, Waltham, MA, USA): 10 mL dH₂O for 40 min followed by 20 min at room temperature. The sections were then washed with TBS-0.5% Tween-20 and incubated with hydrogen peroxide 10% (v/v). Primary antibody SOX2 was diluted 1:50 in antibody diluent OP Quanto (Thermo Fisher, Waltham, MA, USA) and incubated overnight at 4 °C in a humidified chamber. After incubation, slides were washed and

incubated with the secondary antibody HRP polymer (Rabbit/Mouse Dako Real EnVision, Denmark) for 30 min at room temperature. Next, the slides were washed twice with TBS-0.5% Tween-20 and incubated with Substrate Buffer mix with DAB + Chromogen (50X) (Dako Real, Denmark), according to the manufacturer's instructions, for 3 min at room temperature in a humidified chamber. Slides were then washed with water and counterstained with hematoxylin for 2 min and washed again. Finally, the slides were dehydrated in a series of EtOH dilutions, passed through xylene and mounted with permanent mounting medium (Bio-mount HM; Bio-Optica, Milan, Italy).

4.12. SOX2 Overexpression and Knockdown

Cells were seeded in six-well plates at a density of 0.6×10^5 cells/mL and incubated for 24 h at 37 °C and 5% CO₂ in a humidified incubator. For SOX2 overexpression, AGS and Kato III SORE6[−] cells were transiently transfected with a SOX2 expression vector or with the corresponding empty vector (pcDNA3.1) in a 1 µg DNA: 1.5 µL lipofectamine ratio in serum- and antibiotic-free Opti-MEM medium. For SOX2 knockdown, AGS SORE6⁺ and Kato III SORE6⁺ cells were transiently transfected with either a commercial set of three stealth siRNAs directed against human SOX2 (HSS144045, HSS186041, and HSS186042) or an siRNA negative control (CTRL siRNA), all from Invitrogen (Carlsbad, CA, USA). The transfection was performed using Lipofectamine 2000 with 100 nM of siRNAs targeting SOX2 or siRNA negative control (CTRL) in serum- and antibiotic-free Opti-MEM medium. After 24 h, the transfection medium was replaced with DMEM without phenol red supplemented with 10% FBS and 1% sodium pyruvate. Twenty-four hours later, the cells were submitted to annexin V/PI assay and BrdU incorporation assay. The efficiency of SOX2 overexpression and knockdown was verified by Western Blot and qRT-PCR respectively.

4.13. Real-Time2 Profiler PCR Array (RT2PCR)

We used the RT2 ProfilerTM PCR Array Human Cancer Drug Resistance (Qiagen, Hilden, Germany) to identify genes involved in drug resistance that could be differentially expressed between SORE6[−] and SORE6⁺ cells. AGS SORE6⁺ and SORE6[−] total RNA was extracted, quantified and mRNA converted to cDNA using the same procedures previously mentioned. For the PCR, a master mix was prepared, according to the manufacturer's instructions, with 102 µL of a cDNA solution (91 µL of RNase-free water and 20 µL of cDNA), 1350 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 1248 µL of RNase-free water (NZYTech, Lisboa, Portugal). Then, 19 µL of the mix were distributed in each well of a 96-well plate. The reactions were performed in a 7500 Fast Real-Time PCR System using the software v2.0.6 (Applied Biosystems, Foster City, CA, USA). The average of five housekeeping control genes included in the kit was used for normalization of target gene abundance. Data were analyzed by the comparative 2 $\Delta\Delta$ CT method [80].

4.14. Small Molecules High-Throughput Screening

AGS SORE6⁺ and SORE6[−] cells were seeded in 384-well plates (Greiner CELLSTAR[®] 781091) at a density of 5×10^4 cells/mL in RPMI medium supplemented with 10% FBS and allowed to attach overnight. The next day, 1200 compounds from the Prestwick Chemical Library were transferred, one compound per well, using a pintool (V&P Scientific, San Diego, CA, USA) coupled to the MDT head of a JANUS Automated Workstation (PerkinElmer, Waltham, MA, USA) for a final concentration of 8 µM in DMSO. For each plate, four wells were treated with DMSO 0.4% and used as negative controls, and quadruplicate wells were treated with 5-FU (4.5 µg/mL) and used as positive controls. Cells were incubated for 48 h after which they were washed with PBS, fixed with 4% PFA (Alfa Aesar, Haverhill, MA, USA), and stained with Hoechst (1 µg/mL). The image acquisition was performed in an INCell Analyzer 2000 (GE Healthcare, Chicago, IL, USA) with a Nikon 10x/0.45 NA Plan Fluor objective. Four fields of view were acquired per well covering the whole well. Image analysis was executed using the INCell Investigator software (GE Healthcare, Chicago, IL, USA). Briefly, the image analysis workflow consists in the segmentation of the nuclei, from the Hoechst channel, followed by a slight expansion of

the nuclear mask to cover a larger area of the cell. The mean pixel intensity of the GFP channel has been extracted for each individual cell. Spotfire software (TIBCO, Palo Alto, CA, USA) was used to visualize the data, perform quality control of the image segmentation and GFP+/GFP− threshold decision. A custom-made MATLAB script (version R2018, MathWorks, Natick, MA, USA) was developed to automatically extract specific and conditional data of all analyzed cells and per each condition. Hits were identified using thresholds for total cells and GFP positive cells loss higher than three times the standard deviation (3SD) of the mean of the negative controls and loss of GFP positive cells. Primary hit confirmation was performed testing each compound in a dose–response assay (0.125 μ M–16 μ M) in triplicate. For hit validation, compounds were repurchased and tested again in dose–response in cells were seeded in 96-well plates (CellCarrier-96 Ultra, PerkinElmer, Waltham, MA, USA) at a density of 2.5×10^4 cells/mL and incubated for 24 h at 37 °C and 5% CO₂ in a humidified incubator. Then, cells were exposed to the compounds for 48 h and then fixed, stained, and analyzed as previously described for the primary screening. DMSO was used as a negative control and 5-FU used as a positive control.

4.15. Statistical Analysis

Results were expressed as mean $\pm$ SD from at least three independent experiments. Statistical significance was determined by unpaired two-tailed t test or one-way ANOVA followed by post hoc Turkey's test using GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when $p \leq 0.05$.

5. Conclusions

We demonstrated that SOX2 has a prominent role in gastric CSCs and can be used as a marker of these cells. Furthermore, we identified a new drug, monensin, with increased toxicity over gastric CSCs. We showed for the first time that monensin acts on CSCs, paving the way for the clinical testing of this drug in GC patients.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/2/495/s1>. Figure S1: SORE6+ cells are enriched in NOTCH1 and c-MYC and give rise to SORE6− cells in both gastric cancer cell lines. Figure S2: SORE6+ cells have a higher incorporation of BrdU compared to the SORE6− cells and an enhanced ability to form tumors in vivo. Figure S3: SORE6− cells are more sensitive to chemotherapeutic drugs and Kato III SORE6+ cells are more sensitive to monensin than SORE6− cells. Figure S4: Uncropped western blots images corresponding to all the figures in the manuscript.

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

Funding: This research was funded by This work was supported by FEDER-Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020–Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT–Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274); project NORTE-07-0124-FEDER-000029 supported by Norte Portugal Regional Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); and projects POCI-01-0145-FEDER-029017 and POCI-01-0145-FEDER-016390 funded by FCT. Diana Pádua acknowledges FCT for financial support through a PhD fellowship (SFRH/BD/146186/2019).

Acknowledgments: The authors acknowledge Lalage M. Wakefield from NCI, USA for the kind gift of the SORE6-GFP reporter system and the support of the i3S Scientific Platforms BioSciences Screening (BS) and Advanced Light Microscopy (ALM), members of the national infrastructure PPBI-Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122).

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials: A SOX2 Reporter System Identifies Gastric Cancer Stem-Like Cells Sensitive to Monensin

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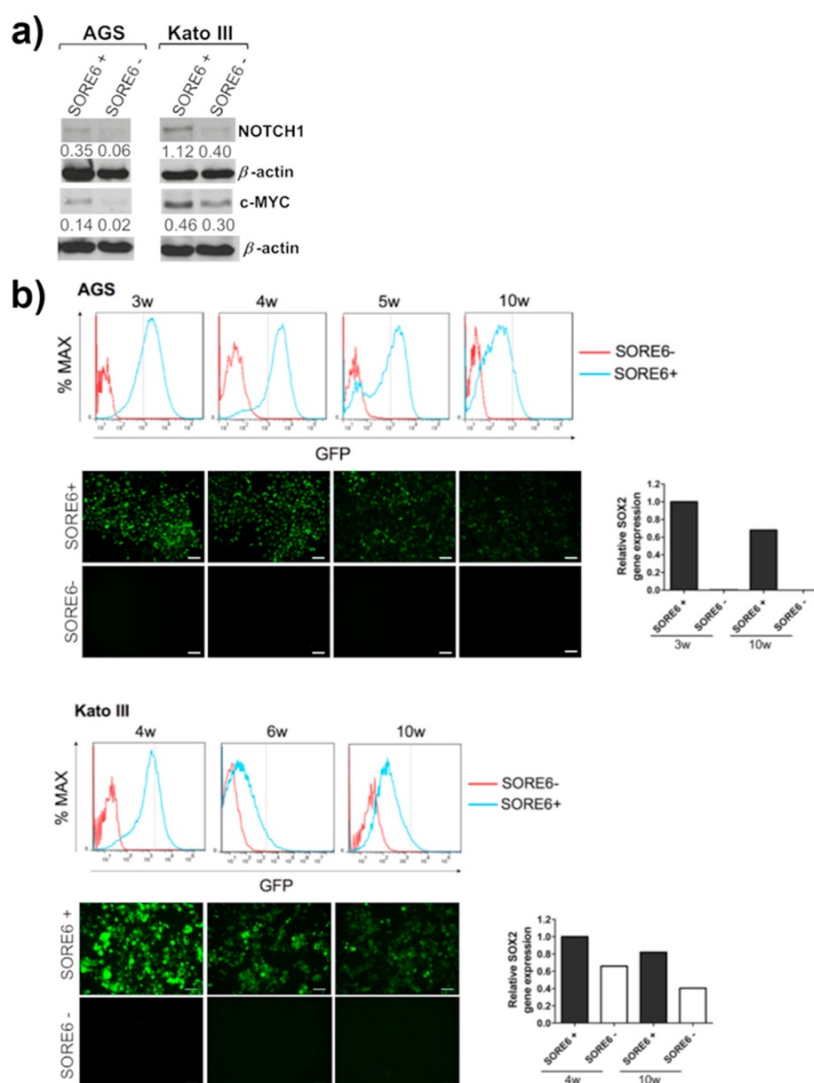


Figure S1. SORE6+ cells are enriched in NOTCH1 and c-MYC and give rise to SORE6- cells in both gastric cancer cell lines. **a)** Western blot exhibiting NOTCH1 and c-MYC expression in SORE6+ and SORE6- cells from both cell lines. β -actin was used as an internal control. Numbers below every western blot picture represent semi-quantitative analysis of each line (intensity ratio: “gene of interest”/ β -actin). Pictures show cropped areas of western blots, the whole images are included in the Supplementary Materials. **b)** FACS plot of AGS SORE6+ cells and AGS SORE6- cells 3, 4, 5 and 10 weeks (w) after sorting followed by fluorescence images of the respective populations at those timepoints, scale bar = 100 mm. SORE6+ cells can regenerate to SORE6- cells while SORE6- cells are always GFP negative. Followed by the analysis of SOX2 expression – intensity ratio - (by western blot) in both SORE6 subpopulations at the initial and final time-point. Results are mean $\pm$ SD. Significant differences

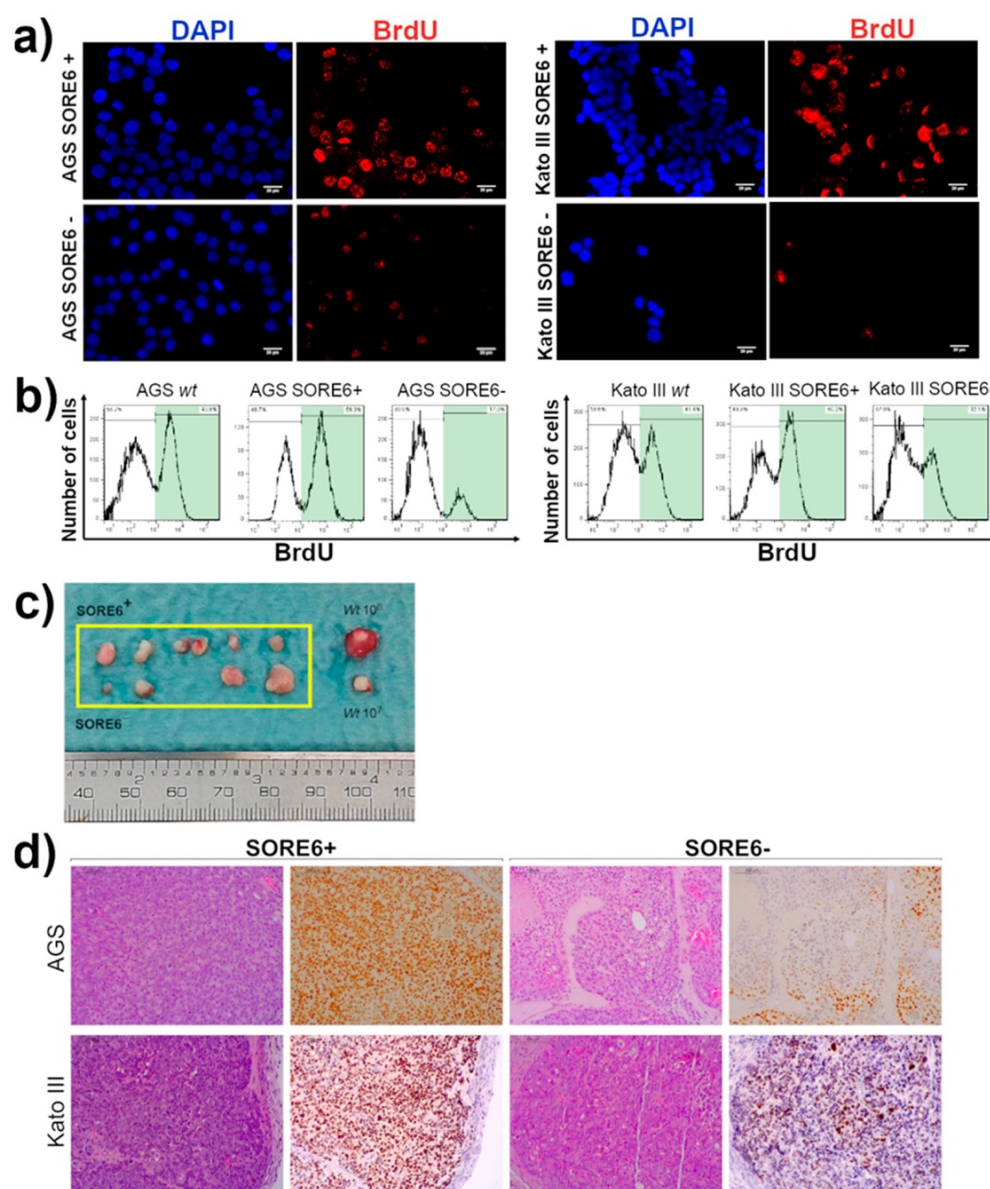


Figure S2. SORE6+ cells have a higher incorporation of BrdU compared to the SORE6- cells and an enhanced ability to form tumors in vivo. **a)** Immunofluorescence channels displaying the DAPI and the BrdU staining in AGS and Kato III SORE6 subpopulations. Scale bar = 100 mm. **b)** FACS plot of AGS wt, SORE6+ and SORE6- cells and Kato III wt, SORE6+ and SORE6- cells showing the proliferative activity of the cells. **c)** Representative picture of the tumors obtained (yellow rectangle) after inoculation of six mice with AGS SORE6+ cells (six tumors; above) or AGS SORE6- cells (four tumors; below). **d)** H&E staining and respective immunohistochemical staining for SOX2 from the tumors originated from SORE6+ and SORE6- cells from both cell lines. All tumors exhibited necrotic regions of variable extension. Magnification, 200 ×, scale bar = 100 mm.

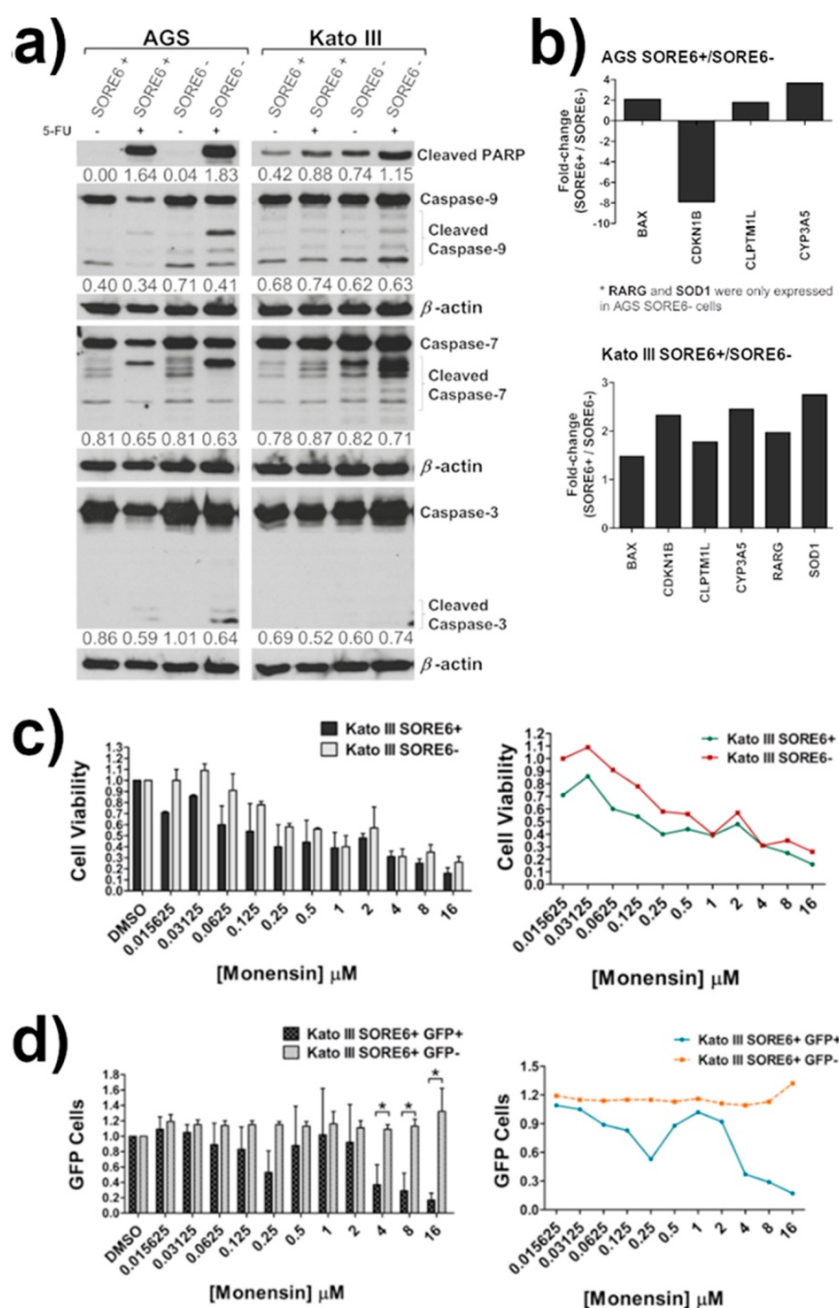
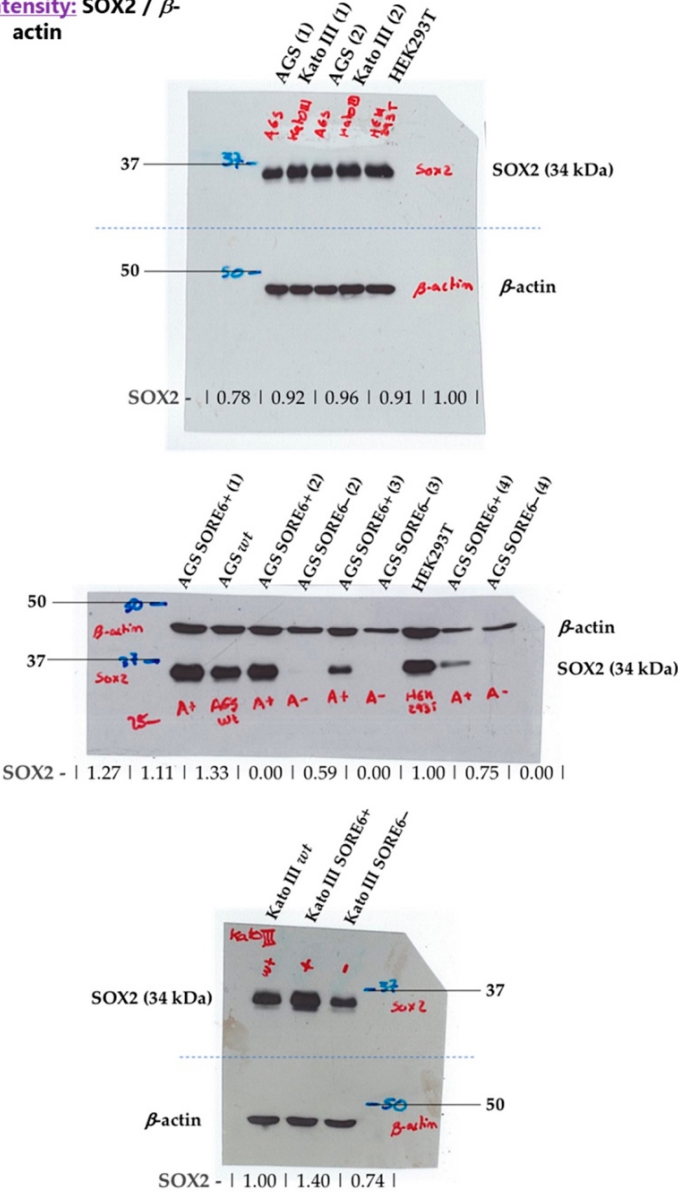


Figure S3. SORE6⁻ cells are more sensitive to chemotherapeutic drugs and Kato III SORE6⁺ cells are more sensitive to monensin than SORE6⁻ cells. a) Western blot showing cleaved PARP, Caspases-9, -7, -3 and cleaved Caspase-9, -7 and -3 expression in SORE6⁺ and SORE6⁻ cells from both cell lines after incubation for 48 h with 5-FU. b-actin was used as an internal control. Numbers below every western blot picture represent semi-quantitative analysis of each line (intensity ratio: "gene of interest"/b-actin). Pictures show cropped areas of western blots, the whole images are included in the Figure S4) Validation, through RT-PCR, in AGS and Kato III SORE6 subpopulations of the most relevant genes that showed a significant fold change, up- or down-regulation, ($p \leq 0.05$) in AGS SORE6⁺ cells compared to AGS SORE6⁻ cells. c) Kato III SORE6⁺ and SORE6⁻ cells viability results after 48h treatment with monensin, normalized for the DMSO. d) Number of GFP⁺ and GFP⁻ cells in the Kato III SORE6⁺ population after 48h treatment with monensin, normalized for the DMSO. Results are mean $\pm$ SD of three independent experiments. Significant differences (* $p \leq 0.05$). .

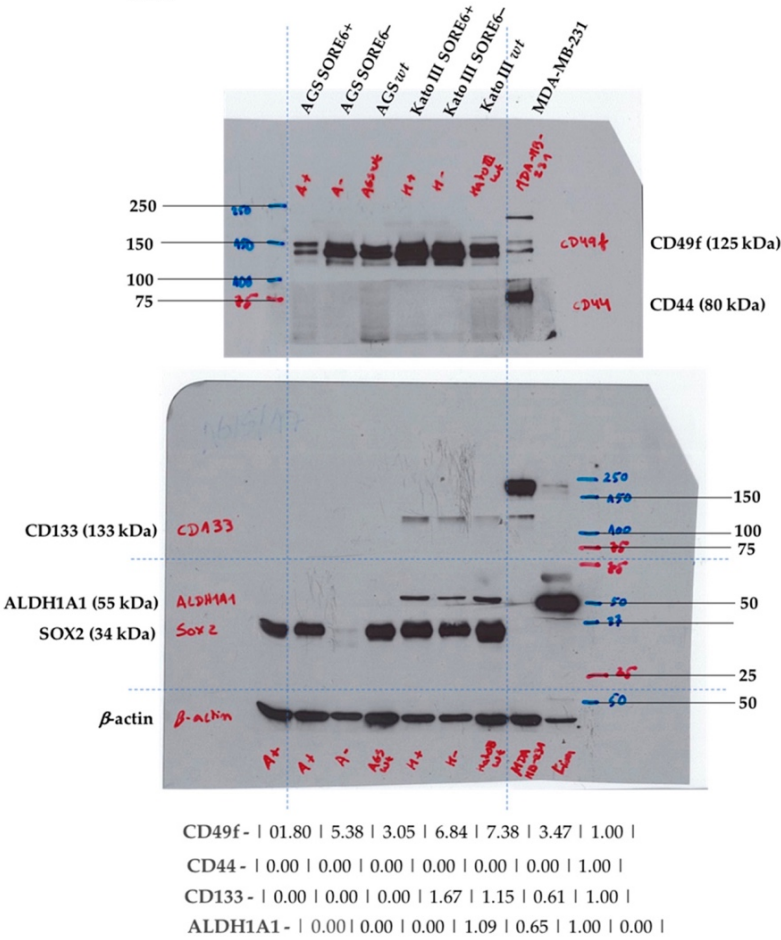
Full unedited gels for Figure 2 a) and d)

Ratio of intensity: SOX2 / β -actin



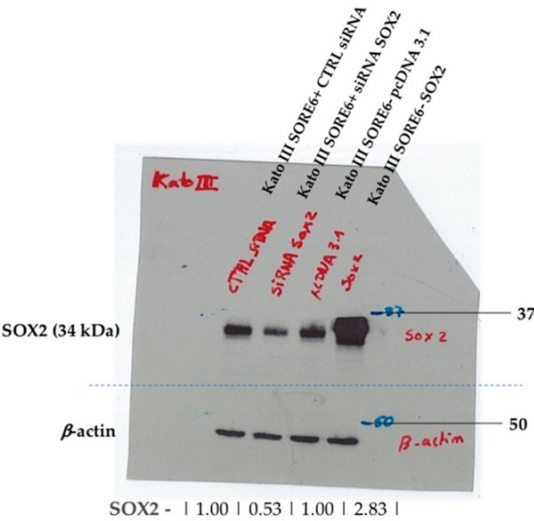
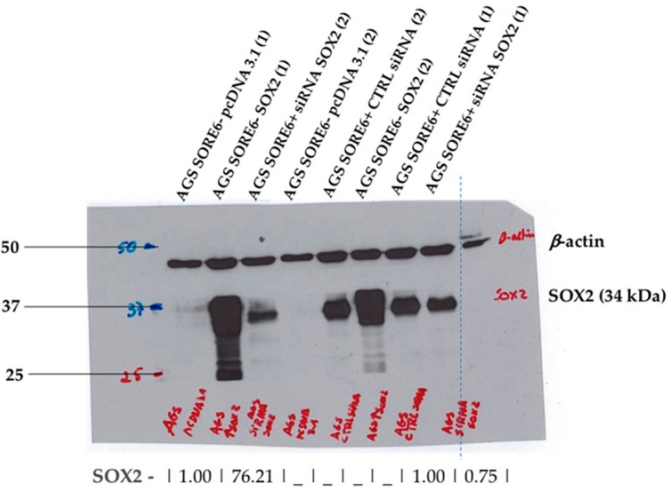
Full unedited gels for Figure 2 e)

Ratio of intensity: CD49f / β -actin
Ratio of intensity: CD44 / β -actin
Ratio of intensity: CD133 / β -actin
Ratio of intensity: ALDH1A1 / β -actin



Full unedited gels for Figure 5 a) and b)

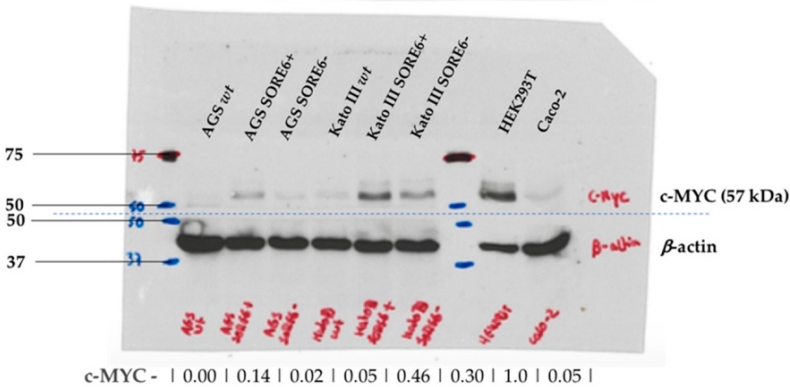
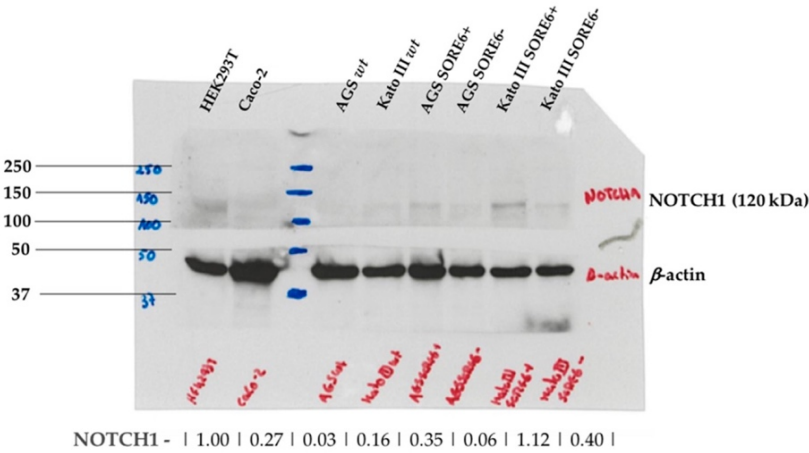
Ratio of intensity: SOX2 / β -actin

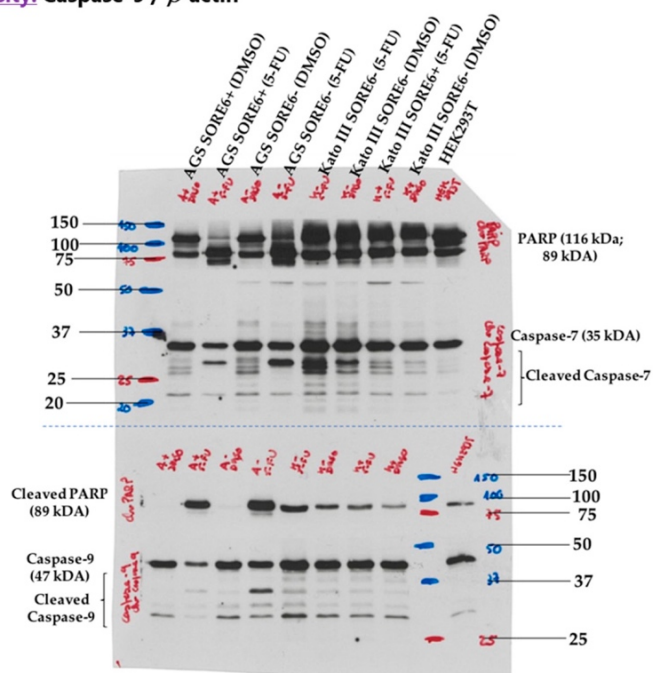


Full unedited gels for Supplementary Figure 1 a)

Ratio of intensity: NOTCH1 / β -actin

Ratio of intensity: c-MYC / β -actin

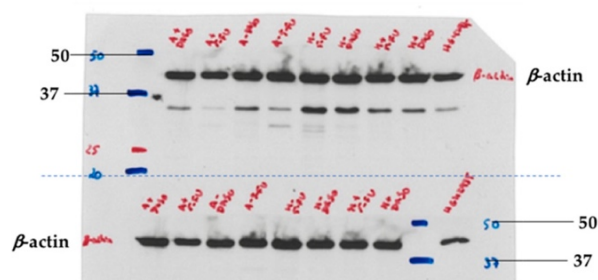


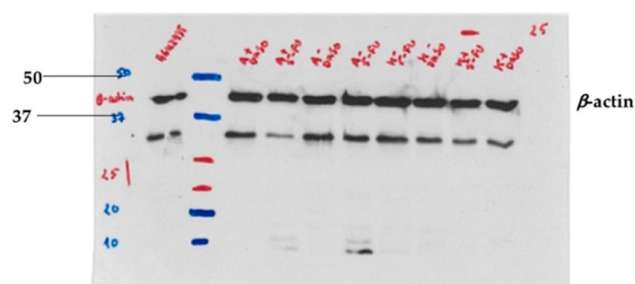
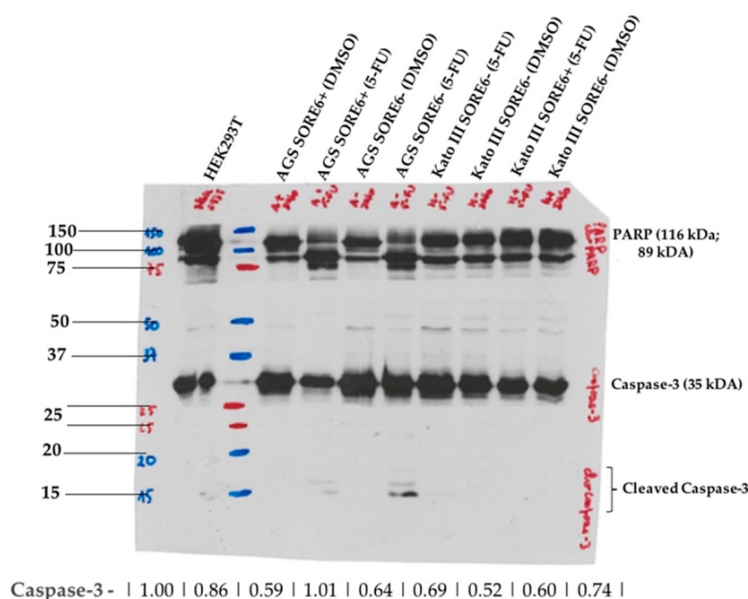
Full unedited gels for Supplementary Figure 3 a)**Ratio of intensity: Caspase-7 / β -****actin****Ratio of intensity: Cleaved PARP / β -****actin****Ratio of intensity: Caspase-9 / β -actin**

Caspase-7 - | 0.81 | 0.65 | 0.81 | 0.63 | 0.78 | 0.87 | 0.82 | 0.71 | 1.00 |

Cleaved PARP - | 0.00 | 1.64 | 0.04 | 1.83 | 0.42 | 0.88 | 0.74 | 1.15 | 1.00 |

Caspase-9 - | 0.40 | 0.34 | 0.71 | 0.41 | 0.68 | 0.74 | 0.62 | 0.63 | 1.00 |



Full unedited gels for Supplementary Figure 3 a)**Ratio of intensity: Caspase-3 / β -actin****Figure S4.** Uncropped western blots images corresponding to all the figures in the manuscript.

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CHAPTER III

HIGH-THROUGHPUT DRUG SCREENING REVEALED THAT CICLOPIROX OLAMINE CAN ENGENDER GASTRIC CANCER STEM-LIKE CELLS

This chapter contains the following publication:

Pádua, D., Figueira, P., Pinto, M., Maia, A. F., Peixoto, J., Lima, R. T., Pombinho, A., Pereira, C. F., Almeida, R., & Mesquita, P. (2023). *High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-like Cells*. *Cancers*, 15(17), 4406.

<https://doi.org/10.3390/cancers15174406>

In Chapter II, we showed that by using the SORE6-GFP reporter system we were able to isolate and characterize two gastric cancer cell populations, SORE6⁻ and SORE6⁺, based on GFP expression. Contrary to SORE6⁻ cells, SORE6⁺ cells exhibited the expected cancer stem-like features and an enrichment in several molecules, including SOX2. Our results also showed that SOX2 expression significantly interferes with cell proliferation and resistance to 5-FU. Thus, our data clearly showed that SOX2 expression has a significant role in some crucial characteristics of gastric CSCs. Therefore, using the SORE6-GFP reporter system embedded in SORE6⁻ cells, we decided to reprogram these cells to make them GFP⁺, i.e., cancer stem-like cells, and explore which TFs are associated with the induction of SOX2 expression. As mentioned in Chapter I, cellular reprogramming using TFs is not very efficient and, in this case, could hinder our goal of identifying TFs associated with the cancer stem-like phenotype. So, we resort to reprogramming using small molecules, which has superior efficiency and reproducibility. To this end, we performed a high-throughput screening of small molecules to find compounds capable of activating the SORE6-GFP reporter system incorporated in SORE6⁻ cells, that is, capable of inducing the expression of SOX2 and/or OCT4 to activate GFP expression. We also took the opportunity to explore the cellular mechanisms involved in the reprogramming of non-CSCs into CSCs.

In this Chapter, *High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-Like Cells*, we report that the antifungal agent ciclopirox olamine (CPX), an iron chelator, can reprogram gastric non-CSC cells into cancer stem-like cells. Although CPX has a potent cytotoxic effect on gastric cancer cells, depending on the dose and exposure time, it can also promote the biogenesis of gastric CSCs. Our data showed that CPX treatment was able to activate the reporter system incorporated into SORE6⁻ cells, inducing/increasing SOX2 expression. It also increased the expression of other molecules, including C-MYC, HIF-1 α , KLF4 and HMGA1. Treatment for 48 h with 4 or 8 μ M CPX leads to cellular senescence, which has been linked to CSCs properties, and induces a metabolic shift from OXPHOS to glycolysis, a potential pathway used by gastric CSCs to obtain energy. Our results also revealed that the percentage of GFP⁺ cells decreased over time, which may be associated with one of the characteristics of CSCs, asymmetric division. We also found that a second exposure of cells to CPX increased the conversion efficiency of GFP⁻ cells into GFP⁺, showing that these cells, recovered from a first treatment, present enhanced cellular plasticity and resistance to CPX. Furthermore, we revealed that the mechanism underlying CPX-mediated cellular reprogramming is similar to that of cobalt chloride (CoCl₂), a hypoxia mimetic agent.



Article

High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-like Cells

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Citation: Pádua, D.; Figueira, P.; Pinto, M.; Maia, A.F.; Peixoto, J.; Lima, R.T.; Pombinho, A.; Pereira, C.F.; Almeida, R.; Mesquita, P. High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-like Cells. *Cancers* **2023**, *15*, 4406. <https://doi.org/10.3390/cancers15174406>

Academic Editor: Rosalba D'Alessandro

Received: 25 July 2023

Revised: 28 August 2023

Accepted: 1 September 2023

Published: 3 September 2023



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Simple Summary: Cancer stem cells (CSCs) are thought to be involved in tumor initiation and recurrence, which makes them pivotal therapeutic targets. However, the molecular circuits behind CSC characteristics are not fully understood and the low number of CSCs is an obstacle for conducting tests that explore their properties. Thus, increasing the number of these cells via small molecule-mediated cellular reprogramming seems a valid alternative tool. Using the SORE6-GFP reporter system, based on SOX2/OCT4 activity and incorporated in gastric non-CSCs, we performed a high-throughput drug screen to identify small molecules capable of converting non-CSCs into CSCs. Our results show that ciclopirox olamine (CPX) is able to reprogram gastric cancer cells to a stem-like phenotype via SOX2 expression, reprogram cell metabolism, and induce senescence. Furthermore, these results suggest that the CSC phenotype is a resistance mechanism to CPX treatment, which is being considered as a repurposing drug for cancer treatment.

Abstract: Cancer stem cells (CSCs) are relevant therapeutic targets for cancer treatment. Still, the molecular circuits behind CSC characteristics are not fully understood. The low number of CSCs can sometimes be an obstacle to carrying out assays that explore their properties. Thus, increasing CSC numbers via small molecule-mediated cellular reprogramming appears to be a valid alternative tool. Using the SORE6-GFP reporter system embedded in gastric non-CSCs (SORE6[−]), we performed a high-throughput image-based drug screen with 1200 small molecules to identify compounds capable of converting SORE6[−] to SORE6⁺ (CSCs). Here, we report that the antifungal agent ciclopirox olamine (CPX), a potential candidate for drug repurposing in cancer treatment, is able to reprogram gastric non-CSCs into cancer stem-like cells via activation of SOX2 expression and increased expression of C-MYC, HIF-1 α , KLF4, and HMGA1. This reprogramming depends on the CPX concentration and treatment duration. CPX can also induce cellular senescence and the metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis. We also disclose that the mechanism underlying the cellular reprogramming is similar to that of cobalt chloride (CoCl₂), a hypoxia-mimetic agent.

Keywords: gastric cancer; cancer stem cells; ciclopirox; cellular reprogramming; transcription factors; cellular senescence; metabolic reprogramming; cobalt chloride

1. Introduction

Gastric cancer is the fifth most commonly diagnosed cancer and the fourth deadliest cancer in the world [1]. The search for new therapeutic targets has led to the recognition of CSCs as cells responsible for tumor progression, recurrence, and resistance to therapy [2–4]. CSCs were first identified in acute myeloid leukemia and have since been identified in most cancers, including gastric cancer [5–7]. CSCs constitute a small cell population in the tumor bulk with self-renewal and proliferation capacities and are resistant to various therapies [7,8]. Additionally, studies have shown that these stem-like characteristics are maintained by the adoption of different mechanisms driven by senescence and cellular plasticity [9,10].

Recently, we have transduced two gastric cancer cell lines with the SORE6–GFP reporter system developed by Tang et al., which relies on the activity of the transcription factors (TFs) SOX2 and OCT4 to drive the expression of the GFP reporter, i.e., after incorporation of the reporter system into cells, those that express sufficient levels of SOX2 and/or OCT4 will activate GFP expression, while those that do not express sufficient levels of these TFs remain GFP-negative [7,11]. Two different subpopulations were isolated by fluorescence-activated cell sorting (FACS) and characterized in each gastric cancer cell line: a small subpopulation expressing GFP, SORE6+, which exhibited the expected cancer stem-like features responsible for tumor progression and therapy resistance and can be considered CSCs, in opposition with a GFP-negative subpopulation, SORE6– (non-CSCs). Additionally, SORE6+ cells are enriched in several stemness-related molecules including SOX2, C-MYC, and Notch1, when compared with SORE6– cells [7]. However, the molecular circuitries of CSCs that link stemness with therapy resistance and patient prognosis are not well understood.

Cellular reprogramming is an interesting approach for converting cancer cells into CSCs to produce a greater CSC population for experimental manipulation and to explore the molecular mechanisms that induce the CSC phenotype [12,13]. Reprogrammed CSCs exhibit features and genetic profiles similar to those of CSCs and serve as a useful platform for investigating their origin and molecular functions [13]. Cellular reprogramming of mouse and human somatic cells to a stem-like phenotype has been accomplished by ectopic expression of a cocktail of TFs, such as SOX2, OCT4, KLF4, and C-MYC or SOX2, OCT4, LIN28, and NANOG [14–16]. However, the efficiency of cellular reprogramming using TFs remains low because of genetic and epigenetic barriers [14,15]. To overcome this difficulty, small molecules have been tested and used to increase the efficiency of cellular reprogramming and/or to replace some of the main TFs [17,18]. For example, valproic acid, a histone deacetylase inhibitor, increased the efficiency of TF-mediated cellular reprogramming, showing that chromatin modification may be one of the key rate-determining steps during cellular reprogramming [19]. Although most small molecule screenings have been directed towards the discovery of compounds that are capable of killing CSCs, such as salinomycin and monensin [7,20,21], small molecule-mediated cellular reprogramming appears to be a relevant alternative tool for obtaining a larger population of CSCs. It also has appealing advantages including low cost, simplicity, and reversibility [22].

Via high-throughput screening of small molecules, we identified a compound capable of reprogramming gastric cancer cells into CSCs. Here, we report that the small molecule CPX, an iron chelator, has a potent cytotoxic effect on gastric cancer cells, but it can also reprogram gastric non-CSCs to a cancer stem-like cell phenotype, depending on the dose and treatment duration. CPX can promote the metabolic shift from OXPHOS to glycolysis, induce cellular senescence, and increase the expression of several TFs associated with stemness features such as SOX2, C-MYC, HIF-1 α , KLF4, and HMGA1. Furthermore, we showed that the mechanism underlying this cellular reprogramming is similar to that of CoCl₂, a hypoxia-mimetic agent that stabilizes the expression of HIF-1 α .

2. Materials and Methods

2.1. Cell Culture

Four human gastric carcinoma cell lines (AGS SORE6[−], AGS SORE6⁺, Kato III SORE6[−], and Kato III SORE6⁺) established under the scope of previous work [7] were used in this study. Furthermore, the human gastric carcinoma cell lines AGS (ATCC, CRL-1739, Manassas, VA, USA) and Kato III (ATCC, HTB-103, Manassas, VA, USA), from which the SORE6 cell lines were derived, were also used, as well as the human embryonic kidney cell line, HEK293T (kindly provided by Dr. João Relvas from Glial Cell Biology Group, i3S, Porto, Portugal). All gastric cancer cell lines were cultured in RPMI medium 1640 with 25 mM HEPES and GlutaMAX-1 (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France). The HEK293T cell line was cultured in DMEM with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Life Technologies, Thermo Fisher) supplemented with 10% FBS. All cells were maintained at 37 °C and 5% (*v/v*) CO₂. When the cell cultures reached a confluence of around 80% they were trypsinized with 0.05% Trypsin-EDTA (1X) (Gibco, Life Technologies, Thermo Fisher) and sub-cultured or used in cell-based assays.

2.2. High-Throughput Screening of the Prestwick Chemical Library

AGS SORE6[−] cells [7] were seeded in 384-well plates (CELLSTAR 781091, Greiner, Kremsmünster, Austria) at a density of 5×10^4 cells/mL in RPMI medium 1640 with 25 mM HEPES and GlutaMAX-1 supplemented with 10% FBS. High-throughput screening of 1200 compounds from the Prestwick Chemical Library was performed as described in [7]. Briefly, AGS SORE6[−] cells were exposed to 1200 small molecules, one compound per well, for 48 h at a final concentration of 8 μ M in dimethyl sulfoxide (DMSO) (Applchem, IL, USA). Cells treated with 0.4% DMSO were used as a negative control. The cells were then fixed with 4% paraformaldehyde (PFA) (Alfa Aesar, Haverhill, MA, USA) and stained with Hoechst (1 μ g/mL). The INCell Analyzer 2000 (GE Healthcare, Chicago, IL, USA) was used to acquire the images, and image analysis was performed using INCell Investigator software (GE Healthcare). Both image acquisition and analysis were conducted as described in [7]. The image acquisition was performed in an INCell Analyzer 2000 with a Nikon 10 $\times$ /0.45 NA Plan Fluor objective. Four fields of view were acquired per well covering the whole well. Image analysis was executed using the INCell Investigator software. Briefly, the image analysis workflow consists of the segmentation of the nuclei, from the Hoechst channel, followed by a slight expansion of the nuclear mask to cover a larger area of the cell. The mean pixel intensity of the GFP channel has been extracted for each individual cell. Spotfire software (TIBCO, Palo Alto, CA, USA) was used to visualize the data and perform quality control of the image segmentation and GFP⁺/GFP[−] threshold decision. Additionally, a custom MATLAB script (R2018 version, MathWorks, Natick, MA, USA) was generated to automatically obtain specific and conditional data from all the analyzed cells for each condition. Hits were identified using the SORE6[−]-GFP reporter system as a readout for successful cellular reprogramming, indicating that a significant increase in GFP expression in AGS SORE6[−] cells (GFP-negative) was considered a potential hit. For the final hit validation, the compound was purchased and tested again.

2.3. Assessment of Ciclopirox Activity and Cytotoxicity

AGS SORE6[−] cells were seeded in 96-well plates (CellCarrier-96 Ultra, PerkinElmer, Waltham, MA, USA) at a density of 5×10^4 cells/mL and allowed to attach overnight. Cells were then incubated for 48 h with various concentrations (0.125–16 μ M) of ciclopirox olamine (ciclopirox ethanolamine; ciclopirox; CPX) (Abcam 143286, Cambridge, UK) or 0.4% DMSO. To assess cell viability and GFP expression, cells were fixed, stained, and analyzed using the INCell Analyzer 2000 and INCell Investigator software, as described in the Materials and Methods High-throughput screening of the Prestwick chemical library. To further explore the effect of CPX on cell viability and activation of the SORE6[−]-GFP reporter system, AGS SORE6[−] cells were seeded in 12-well plates at a density of 1×10^5 cells/mL

and allowed to settle for 24 h. Then, cells were exposed to 4 or 8 μM CPX for 2, 6, 12, 24, or 48 h. For cytotoxicity assessment, culture media was discarded, adherent cells were washed with phosphate-buffered saline (PBS) 1x (grisip, Oporto, Portugal), and 50 μL of PrestoBlue Cell Viability Reagent 1x (ThermoFisher Scientific) prepared in RPMI complete medium was added. Cells were incubated for 1 h and 10 min at 37 °C in a 5% CO_2 humidified atmosphere, protected from light. The absorbance was measured at 560 nm (using 600 nm as a reference wavelength) using a BioTek Synergy 2 multi-mode microplate reader (Biotek, Winooski, VT, USA). GFP expression was evaluated using a FACSCanto II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). The data were analyzed using FlowJo software (version 7.6.1). Cells treated with 8 μM CPX for 24 or 48 h were left in culture for a total of 5 weeks. Every week, cells were collected by trypsinization and centrifuged at 350 rcf for 5 min at room temperature (RT), and cell pellets were used for Western blot and flow cytometry analysis of GFP expression. AGS SORE6– cells and Kato III SORE6– cells were also seeded in 6-well plates at a density of 1.5×10^5 cells/mL and 2×10^5 cells/mL, respectively, and allowed to attach overnight. Cells were then incubated with 2, 4, 8, or 16 μM CPX or 0.4% DMSO for 48 h. After, cells were collected, and cell pellets were used for Western blot and/or real-time PCR analysis. Cells treated with DMSO were used as a negative control. CPX effect was also assessed in CPX-recovered cells. For this, four weeks after exposure to DMSO or 4 or 8 μM CPX, AGS SORE6– and Kato III SORE6– recovered cells were plated in 12-well plates at a density of 1×10^5 cells/mL in RPMI with 10% FBS and allowed to attach overnight. Cells were then exposed for 48 h to 8 μM CPX. For the cytotoxicity assessment, the PrestoBlue assay was performed as described above. The cells were collected and used for flow cytometry and Western blot analyses. Cells treated with DMSO were used as a negative control.

2.4. Protein Extraction and Western Blot

The preparation of whole cell extracts and protein quantification were conducted as detailed in [7]. Briefly, 30 μg of total protein extract was separated by standard SDS-PAGE using the Precision Plus Protein Standard Dual Color protein marker (Bio-Rad, Hercules, CA, USA). Subsequently, proteins were transferred to a nitrocellulose membrane (Amersham, GE Healthcare). The membranes were blocked with either 5% non-fat milk or 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) in tris-buffered saline (TBS)-1% Tween-20 (Sigma-Aldrich) for 1 h at RT. Membranes were incubated overnight at 4 °C with the following primary antibodies: SOX2 (diluted 1:500; Cell Marque, Sigma-Aldrich; APCM-371R-15), C-MYC (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA; (D84C12) #5605), HMGA1 (diluted 1:500; Abcam, Cambridge, UK; ab252930), and β -actin (diluted 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA; sc-47778). After, membranes were washed with TBS-1% Tween-20 and incubated for 1 h at RT with the respective HRP-conjugated secondary antibody: goat anti-rabbit IgG (HRP) (diluted 1:10,000; Cell Signaling Technology, Danvers, MA, USA; #7074) or goat anti-mouse IgG (HRP) (diluted 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA; sc-2005). Signal detection was achieved using the ECL detection kit (Amersham, GE Healthcare). β -actin was used as an internal control.

2.5. RNA Extraction and Real-Time PCR

Total RNA extraction using TRI Reagent (Sigma-Aldrich; T9424) was performed according to the manufacturer's protocol instructions. RNA concentration and quality were evaluated using a NanoDrop ND-1000 spectrometer (V3.5.2 Software). Total RNA (1 μg) was converted to cDNA in a thermocycler (Bio-Rad, Hercules, CA, USA) in a final reaction volume of 20 μL , containing 1 μL of random primers (100 ng/ μL) (Invitrogen, Carlsbad, CA, USA), 1 μL of dNTPs (10 mM) (Invitrogen), 2 μL of 10 $\times$ Reaction Buffer (NZYTech, Lisboa, Portugal), 0.2 μL of RNaseOUT (40 U/ μL) (Invitrogen), and 0.5 μL of NZY Reverse Transcriptase (200 U/ μL) (NZYTech) in DEPC-treated water (Invitrogen). For the PCR, each reaction was prepared as described in [7]. Primer sequences are shown in Table S1.

The reactions were executed in a 7500 Fast Real-Time PCR System using the software v2.0.6 (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling conditions: 40 cycles of 15 s at 95 °C for denaturation, plus 1 min at 60 °C for annealing and a melting curve program (60–95 °C) with continuous fluorescence measurement. Each reaction was performed in triplicate. Negative controls (without cDNA) were included on each plate and the 18S ribosomal RNA (rRNA) was used as a housekeeping gene. The data were analyzed using the $2^{-\Delta\Delta CT}$ method [23].

2.6. Evaluation of Cobalt Chloride (CoCl₂) Activity and Cytotoxicity

AGS SORE6– and Kato III SORE6– cells were seeded in 12-well plates at a density of 1×10^5 cells/mL and 1.5×10^5 cells/mL, respectively, and allowed to settle for 24 h. Afterward, cells were incubated with 100, 200, or 300 µM CoCl₂ for 48 h. Cells were collected and cell pellets were used for Western blot and/or real-time PCR and flow cytometry analysis of GFP expression. Cytotoxicity assessment was performed using PrestoBlue Cell Viability Reagent 1x as described above. Untreated cells (only incubated with complete medium) were used as a negative control.

2.7. Clonogenic Assay

AGS SORE6– cells and Kato III SORE6– cells were treated with CPX or CoCl₂ or were plated in 6-well plates at a density of 300 cells per well and 1500 cells per well, respectively. Cells were left undisturbed for 10 days at 37 °C and 5% (v/v) CO₂, except for the fifth day, when the medium was changed. After 10 days, colony formation was visualized by staining the cells with a 1% crystal violet solution diluted in PFA for 30 min. The number of colonies per condition was assessed with the ImageJ software.

2.8. Seahorse Real-Time ATP Rate Assay

SORE6– cells were seeded in Agilent Seahorse XF24 24-well cell culture microplates at a density of 6×10^4 cells/mL and incubated with RPMI medium for 24 h. Then, cells were treated with CPX (4 µM and 8 µM) or CoCl₂ (100 µM, 200 µM, and 300 µM) for 48 h. After, cells were washed and incubated with XF DMEM Medium (DMEM with 5 mM HEPES and without phenol red, sodium bicarbonate, glucose, pyruvate and L-glutamine, pH = 7.4; Agilent Technologies, Santa Clara, CA, USA) supplemented with 10 mM of XF glucose, 2 mM of XF L-glutamine and 1 mM of XF pyruvate, at 37 °C for 1 h in the absence of CO₂. Next, the real-time ATP rate assay was performed using the Seahorse XF Real-Time ATP Rate Assay Kit (Agilent Technologies) according to Agilent's recommendations, on the Seahorse XFe24 analyzer (Agilent Technologies). There, the baseline oxygen consumption rate (OCR) was measured, followed by sequential OCR measurements via the sequential injection of Oligomycin and Rotenone/Antimycin A. The ratio between the ATP production rate in the glycolytic pathway (glycoATP) and the ATP production rate from mitochondrial oxidative phosphorylation (mitoATP)—ATP rate index—was used as a parameter to identify changes in metabolic phenotype OCR values. Assays were normalized to the total number of cells in each well and analyzed with Agilent Seahorse Analytics.

2.9. Senescence-Associated β-Galactosidase (SA-β-gal) Assay

Cells were plated in 12-well plates at a density of 1×10^5 cells/mL in RPMI with 10% FBS and allowed to attach overnight. Cells were treated with CPX (4 and 8 µM) or CoCl₂ (100, 200, and 300 µM) for 48 h. After, cells were washed with PBS 1× and fixed with 4% PFA, for 5 min at RT. Subsequently, cells were incubated for 16 h at 37 °C in the absence of CO₂ in fresh SA-β-gal staining solution prepared as in Dimri et al. [24]. Senescent cells were counted using the ImageJ software (a minimum of 100 cells per condition was considered). Cells treated with DMSO and untreated cells were used as negative controls for CPX and CoCl₂, respectively.

2.10. Cell Proliferation Assay

Cells were cultured as described in [7]. For cell proliferation analysis, the BrdU incorporation protocol was executed as detailed [7]. The percentage of BrdU-positive cells was assessed using a FACSCanto II flow cytometer. The data were analyzed using FlowJo software (version 7.6.1). BrdU incorporation was also observed by immunofluorescence, as in [7]. The coverslips were incubated with the primary antibody BrdU (Bu20a; diluted 1:200; #5292; Cell Signaling Technology) overnight at 4 °C. The goat anti-mouse Alexa Fluor 594 (diluted 1:150; A11032, Thermo Fisher Scientific) was used as the secondary antibody.

2.11. Statistical Analysis

The results are expressed as the mean $\pm$ standard deviation (SD) from at least three independent experiments. Statistical significance was determined by unpaired two-tailed *t*-test or one-way ANOVA followed by Tukey's post hoc test using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. CPX Activates the SORE6–GFP Reporter System Embedded in Gastric Non-CSCs

To identify new drugs that could be used to reprogram gastric cancer cells into CSCs, we screened 1200 compounds from the Prestwick chemical library in previously obtained AGS SORE6– cells (non-CSCs) [7] and evaluated whether any compounds could activate the SORE6–GFP reporter system incorporated into these cells (Figure 1A). Since AGS SORE6– cells are GFP-negative, contrary to AGS SORE6+ cells [7], compounds that induced GFP expression were considered hits. Of the 1200 compounds, five appeared to activate GFP expression in AGS SORE6– cells (Figure 1B). Further analysis showed that merbromin (mercurochrome) was a false positive, as it emitted yellow-green fluorescence when excited with blue or violet-blue light [25]. Rebamipide, vorinostat, and topotecan, although inducing over 10% GFP expression, were shown to strongly decrease cell viability (over 98%) (Figure 1B,C). Therefore, from the compounds evaluated, only the small molecule CPX appeared to be a strong candidate for cellular reprogramming. This compound activated the SORE6–GFP reporter system in more than 10% of cells while decreasing cell viability by approximately 89% (Figure 1B–D). To further confirm that CPX was a potential cellular reprogramming agent, dose–response assays were performed to assess cytotoxic effects and induction of GFP-positive cells. After 48 h of treatment, CPX caused a dose-dependent cell viability decrease (Figure 1E). Moreover, increasing CPX concentrations of 4, 8, and 16 μ M induced GFP expression in 3%, 7%, and 11% of the surviving cells, respectively (Figure 1F). To rule out the possibility that the observed GFP expression after CPX treatment was related to its autofluorescence, we incubated AGS wt cells with 8 μ M of CPX for 48 h and confirmed there was no fluorescence increase (Figure S1A). Importantly, we determined that CPX treatment induced the expression of SOX2 in AGS SORE6– cells, which led to the activation of the SORE6–GFP reporter system (Figure 1G). These results are the first indication that CPX plays a promising role in gastric CSC reprogramming via activation of SOX2 expression. Although the highest concentration of CPX (16 μ M) resulted in a higher percentage of GFP-positive cells, its cytotoxic effect was high and therefore this concentration was not evaluated in further experiments.

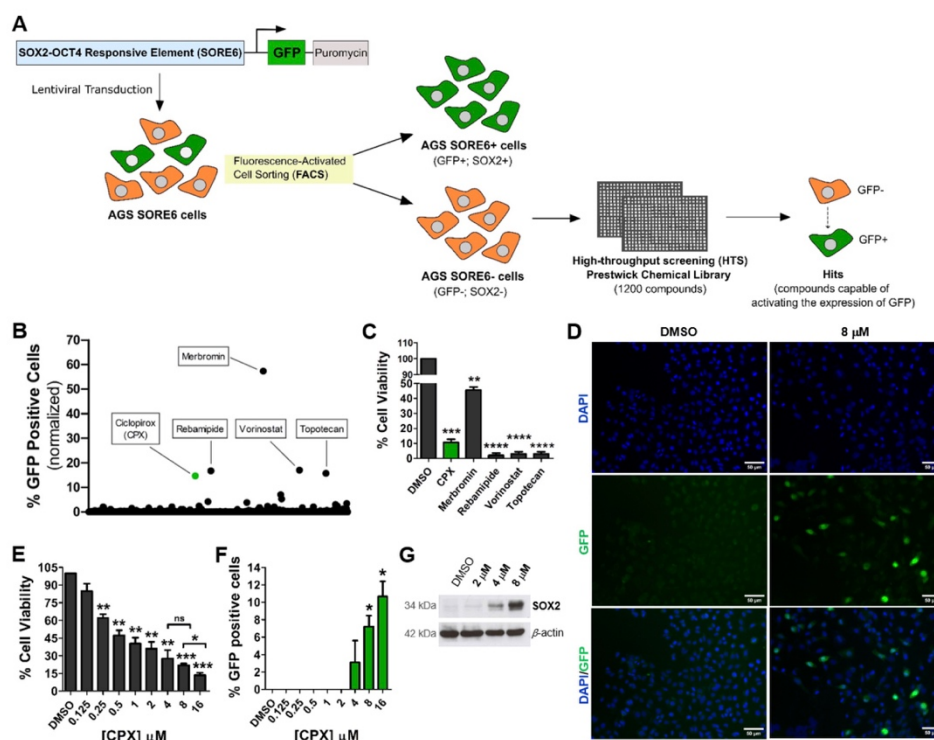


Figure 1. CPX is able to activate the SORE6-GFP reporter system incorporated in the AGS SORE6- cells by inducing SOX2 expression. (A) Scheme of the general approach used to identify compounds capable of transforming SORE6- cells (GFP-negative, non-CSCs) into SORE6+ cells (GFP-positive, CSCs). (B) High-throughput screening results showing the percentage of GFP-positive cells. A total of 1200 small molecules from the Prestwick chemical library were screened. (C) AGS SORE6- cell viability results after 48 h of treatment with CPX, merbromin, rebamipide, vorinostat, and topotecan. (D) Representative immunofluorescence images of reporter system activation after treatment with 8 μ M CPX for 48 h (cells become GFP-positive cells), scale bar = 50 μ m. (E) Percentage of cell viability and (F) percentage of GFP-positive AGS SORE6- cells after incubation for 48 h with increasing CPX concentrations (0.125–16 μ M). The percentage of GFP-positive cells was calculated from viable cells. (G) SOX2 expression in AGS SORE6- cells treated with 2, 4, or 8 μ M CPX for 48 h, evaluated by Western blot, β -actin was used as an internal control, pictures show cropped areas of Western blots, the whole images are included in the Supplementary Materials. DMSO treatment was used as the negative control (CPX solvent/vehicle). Results are mean $\pm$ SD of at least three independent experiments. ns: not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

3.2. Activation of the SORE6-GFP Reporter System via CPX Seems to Be Strongly Associated with Increased SOX2 Expression

To further explore the potential of CPX for cellular reprogramming, we performed a time-course analysis (2, 6, 12, 24, and 48 h) of AGS SORE6- cells treated with 4 and 8 μ M CPX. Cell evaluation at 2 and 6 h treatment, showed no effect of CPX in the activation of the reporter system nor on cell viability (Figure 2A,B). At 12 h treatment, only 8 μ M CPX significantly activated the reporter system (Figure 2A), while at 24 h, treatment with both 4 and 8 μ M CPX induced a significant increase in the percentage of GFP-positive cells (approximately 1% and 4%, respectively). The maximum effect was observed, after 48 h treatment, for both concentrations, being approximately 3% for 4 μ M and 8% for 8 μ M (Figure 2A). Nevertheless, the CPX effect on cellular viability significantly increased over time at both

concentrations (Figure 2B). Next, we evaluated how long the SORE6–GFP reporter system remained active after exposure to CPX. In AGS SORE6– cells treated with 8 μ M for 24 h, GFP expression was maintained for approximately one week, increasing to approximately 3 weeks when cells were treated with CPX for 48 h (Figure 2C). Furthermore, our results suggest an association between the decreased GFP and SOX2 expression (Figure 2D). Thus, activation of the SORE6–GFP reporter system via CPX seems to be strongly associated with the increased SOX2 expression. Since GFP expression lasted longer with the 48 h treatment, this was the exposure time chosen for our further analysis, which included another non-CSC gastric cell line (Kato III SORE6–). Similar to AGS SORE6– cells, Kato III SORE6– cells treatment with CPX (specifically with 8 μ M) significantly activated the reporter system in approximately 4% of the cells (Figure 2E,F). A dose-dependent increase in SOX2 and C-MYC expression was evident in both cell lines (Figure 2G). Although CPX significantly decreased the viability of Kato III SORE6– cells, these cells appeared to be more resistant to CPX treatment than AGS SORE6– cells (Figure 2H). This difference between these two cell lines was also observed in the colony-formation assay (Figure 2I).

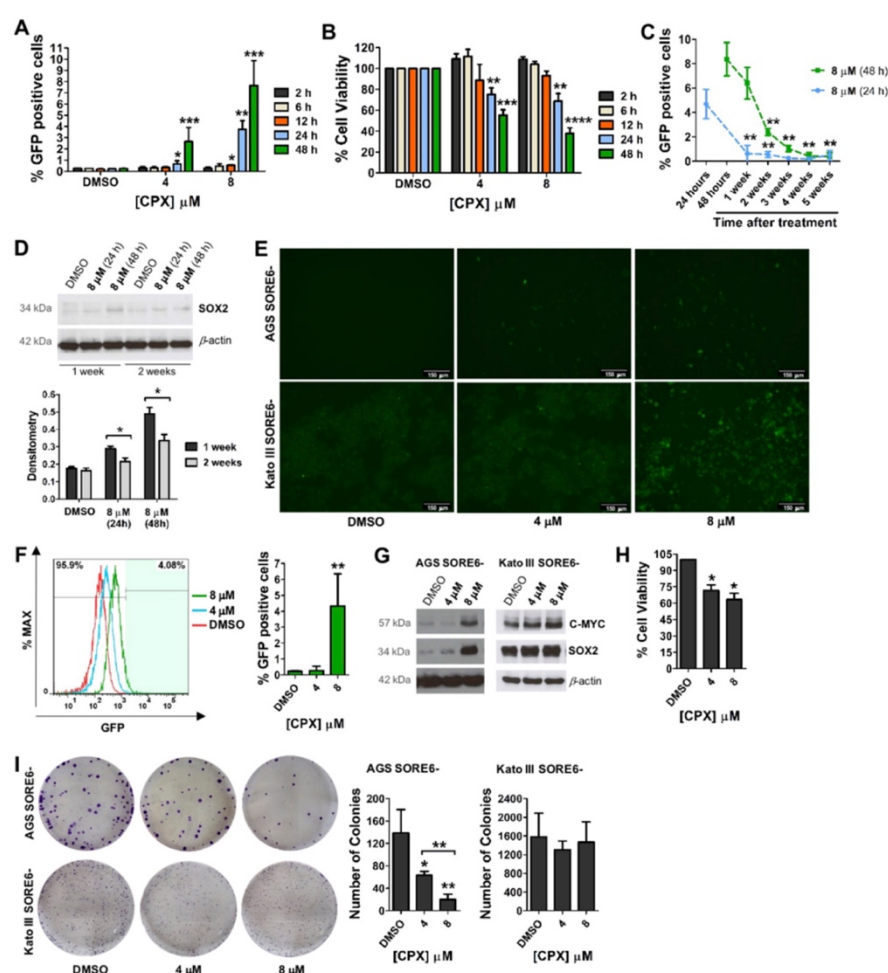


Figure 2. SORE6–GFP reporter system activation by CPX is not permanent and is associated with the consequent loss of SOX2 expression. (A) Percentage of GFP-positive cells and (B) cell viability

after treatment of AGS SORE6– cells with 4 or 8 μ M CPX for 2, 6, 12, 24, and 48 h. (C) Percentage of GFP-positive cells evaluated one to five weeks after treatment of AGS SORE6– cells with 8 μ M CPX for 24 or 48 h. (D) SOX2 expression in AGS SORE6– cells after one and two weeks of treatment with 8 μ M CPX for 24 or 48 h, evaluated by Western blot (and respective densitometry analysis (intensity ratio: “gene of interest”/ β -actin)). (E) Representative fluorescence live cell images of SORE6–GFP reporter system activation in AGS SORE6– and Kato III SORE6– cells after treatment with 4 or 8 μ M CPX for 48 h, scale bar = 150 μ m. (F) Percentage of GFP-positive cells after treatment of Kato III SORE6– cells with 4 or 8 μ M CPX for 48 h, following the respective flow cytometry analysis. (G) SOX2 and C-MYC expression in AGS SORE6– and Kato III SORE6– cells treated with 4 or 8 μ M CPX for 48 h, evaluated by Western blot. (H) Cell viability after treatment of Kato III SORE6– cells with 4 or 8 μ M CPX for 48 h. (I) Colony formation ability of AGS SORE6– and Kato III SORE6– cells after treatment with 4 or 8 μ M CPX for 48 h, representative images of the colonies are shown. β -actin was used as a loading control. Pictures show cropped areas of Western blots, the whole images are included in the Supplementary Materials. DMSO was used as the negative control. Results are mean $\pm$ SD of at least three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

3.3. CPX Promotes a Metabolic Shift from Oxidative Phosphorylation (OXPHOS) to Glycolysis and Induces Cellular Senescence

CSCs show metabolic plasticity, and therefore, one of the relevant characteristics of CSCs is their metabolic phenotype [26]. To assess the effect of CPX on AGS SORE6– cellular metabolism and ATP production rate we used the Seahorse Real-Time ATP rate assay. Briefly, Oligomycin inhibits mitochondrial ATP synthesis by decreasing the rate of oxygen consumption (OCR), which allows quantification of the rate of mitochondrial ATP production. Next, Rotenone/Antimycin A led to complete inhibition of mitochondrial respiration, allowing the assessment of the extracellular acidification rate (ECAR), which allows the determination of glycolytic ATP production. Our analysis showed that 48 h of CPX treatment (4 or 8 μ M) decreased OCR and increased ECAR (Figure 3A,B). CPX treatment significantly decreased the rate of mitochondrial OXPHOS ATP production at both concentrations compared to the control (Figure 3C), whereas the rate of glycolytic ATP production was significantly increased in cells treated with 8 μ M CPX for 48 h (Figure 3C). The ratio of mitochondrial to glycolytic ATP production was calculated and found to be significantly decreased in cells treated with both CPX concentrations (Figure 3D). Additionally, the percentage of glycolysis also increased significantly in the CPX-treated cells (Figure 3E). CPX has been described as an inducer of cellular senescence [27,28]. Senescence has been linked to the promotion of cancer cell reprogramming towards stemness [9]. It can be induced by unrepaired and improper expression of oncogenes, DNA damage, or other cellular stresses [29]. Furthermore, despite the loss of their proliferative capacity, senescent cells have high metabolic activity, preferring energy production from glycolysis rather than OXPHOS [30]. Therefore, we investigated whether CPX triggered senescence in SORE6– cells. Senescence induction was assessed using β -galactosidase (SA- β -gal) activity, a well-established senescence marker [24]. Exposure to 8 μ M CPX for 48 h caused a significant increase in SA- β -gal-labeled cells in both AGS SORE6– (approximately 30%) and Kato III SORE6– cells (around 25%) (Figure 3F). Additionally, CPX treatment also led to an apparent increase in cell size. AGS SORE6– cells treated with 4 and 8 μ M CPX appeared to be larger than control cells and the same was observed in Kato III SORE6– cells treated with 8 μ M CPX (Figure 3F). Furthermore, results from BrdU incorporation by flow cytometry showed that CPX treatment significantly reduced proliferation in both cell lines (Figure 3G).

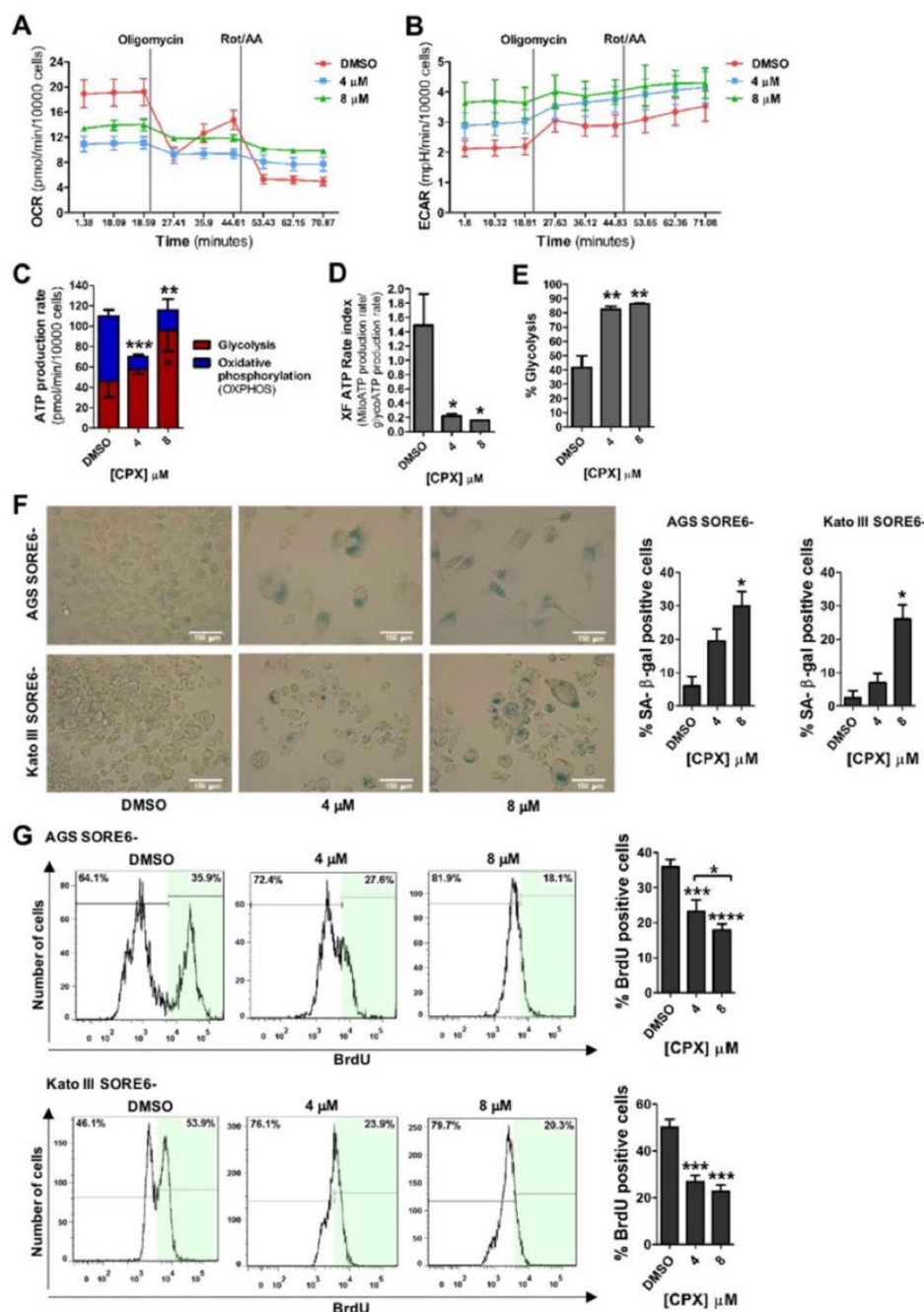


Figure 3. CPX treatment inhibits ATP production by OXPHOS and induces cellular senescence with the consequent loss of proliferative activity. (A) Representative real-time measurements of the kinetic profile of the oxygen consumption rate (OCR), (B) representative real-time measurements of the kinetic profile of the Extracellular Acidification Rate (ECAR), (C) metabolic flux analysis showing quantification of mitochondrial ATP production and glycolytic ATP production, (D) ATP

production rate index calculated from the data showed in C (i.e., mitochondrial ATP production rate/glycolytic ATP production rate), and (E) percentage of glycolysis following treatment with 4 or 8 μ M CPX for 48 h. (F) Percentage of SA- β -gal-positive cells in AGS SORE6– and Kato III SORE6– cells treated with 4 or 8 μ M CPX for 48 h, following representative images of SA- β -gal marking. Scale bar = 150 μ m. (G) Percentage of BrdU positive cells in AGS SORE6– and Kato III SORE6– cells treated with 4 or 8 μ M CPX for 48 h, following representative flow cytometry analysis. DMSO was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

3.4. CPX-Induced Cellular Senescence Is Reversible and Recovered Cells Become More Resistant to CPX and More Prone to SORE6–GFP Reporter System Re-Activation

As mentioned above, senescence drives the reprogramming of cancer cells and promotes CSC generation [9]. Recent studies have suggested that this is achieved by the ability of senescent cells to re-enter the cell cycle to become CSCs, reversing the “irreversible” arrest of cell division and proliferation [31]. Thus, we investigated whether CPX-treated cells could overcome senescence and acquire some characteristics of CSCs, such as increased proliferative activity and drug resistance. AGS SORE6– cells and Kato III SORE6– cells treated for 48 h with 4 or 8 μ M CPX were cultured for 4 weeks. SA- β -gal activity was then evaluated with no significant differences being observed in the percentage of SA- β -gal positive cells when comparing cells recovered from exposure to DMSO or 4 or 8 μ M CPX (Figures 4A and S2A). Interestingly, while evaluation of cells immediately after 48 h treatment with 8 μ M CPX, showed 30% of AGS SORE6– cells and 25% of Kato III SORE6– cells as positive for SA- β -gal, 4 weeks later, only 11% and 7%, respectively, were positive for SA- β -gal, thus revealing a possible reversal of the senescent state (Figures 3F, 4A and S2A). AGS SORE6– cells recovered from CPX treatment also regained the ability to proliferate; specifically, cells recovered from 8 μ M CPX treatment showed significantly improved proliferation activity compared with those recovered from DMSO exposure (negative control) (Figures 4B and S2B). We also re-exposed AGS SORE6– and Kato III SORE6– recovered cells to 8 μ M CPX for 48 h to verify the impact of a new CPX treatment on the activation of the SORE6–GFP reporter system and cell viability. Remarkably, the new treatment with CPX greatly increased the activation of the reporter system. In AGS SORE6– recovered cells, previously treated with 4 μ M or 8 μ M, the re-exposure to 8 μ M CPX for 48 h resulted in 28% and 35% GFP-positive cells, respectively (Figure 4C). In addition, re-exposure to CPX revealed that cells recovered from previous CPX treatment acquired resistance to the compound (Figure 4D). Similar results were obtained in Kato III SORE6– recovered cells (Figure S2C,D). Based on the SORE6–GFP reporter system activation, our results suggest that cells treated with CPX acquire plasticity by dynamically switching between SORE6+ (cancer stem-like cells) and SORE6– (non-CSCs) phenotypes.

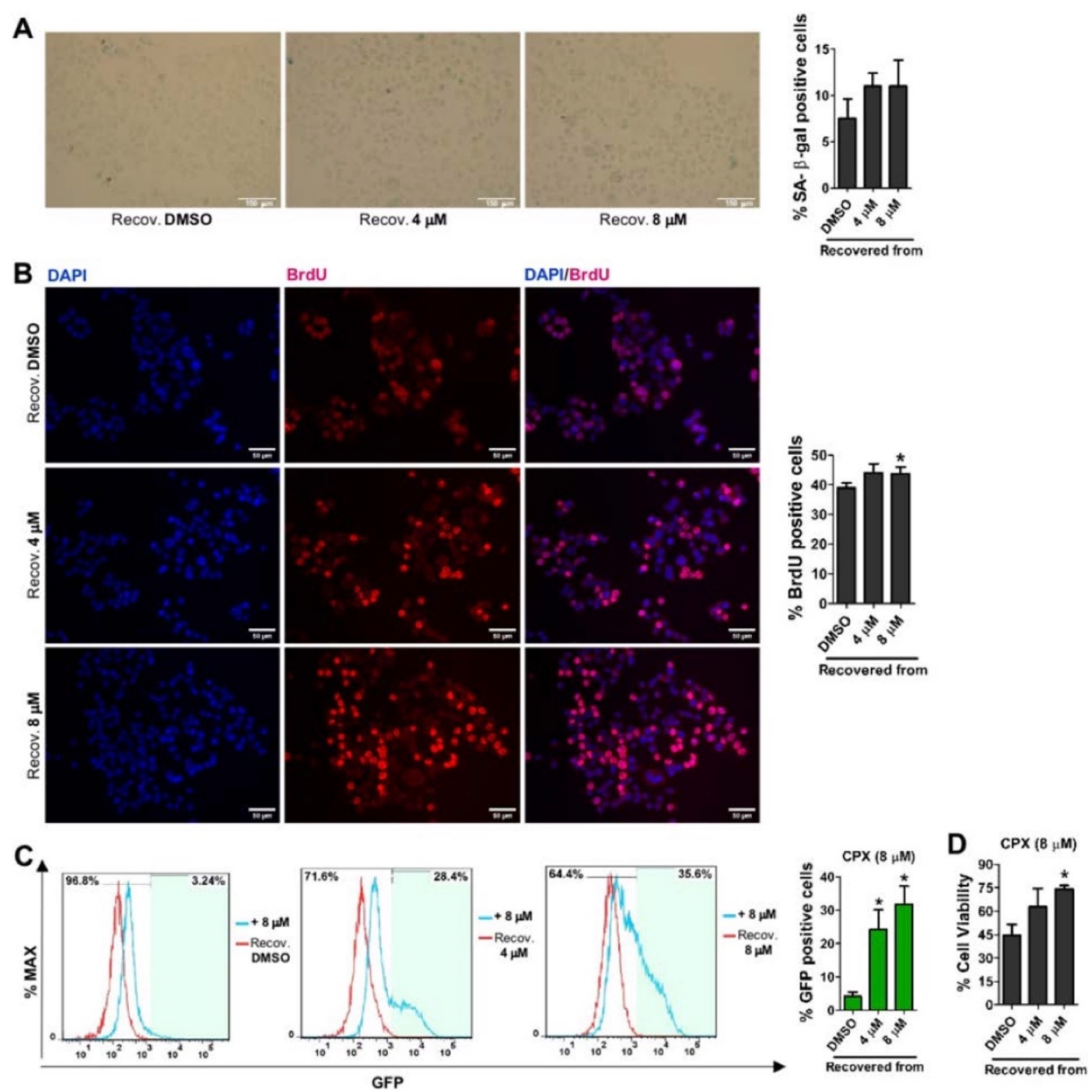


Figure 4. CPX-induced cellular senescence is reversible, and after a new treatment, activation of the SORE6–GFP reporter system and resistance to treatment increase. (A) Percentage of SA- β -gal positive cells in AGS SORE6– cells 4 weeks after recovery from treatment with DMSO, 4 or 8 μ M CPX for 48 h, following the representative images of SA- β -gal marking. Scale bar = 150 μ m. (B) Percentage of BrdU positive cells, evaluated by flow cytometry, in AGS SORE6– recovered cells, following representative immunofluorescence images displaying the BrdU incorporation. Scale bar = 50 μ m. (C) Percentage of GFP-positive cells, following the respective flow cytometry analysis, and (D) cell viability in AGS SORE6– recovered cells after new treatment with 8 μ M CPX for 48 h. DMSO was used as the negative control. Results are mean $\pm$ SD of at least three independent experiments. * $p \leq 0.05$.

3.5. Activation of the Reporter System SORE6-GFP by CPX Has a Mechanism Similar to That of the Hypoxia Mimetic CoCl₂

CPX has been described as a HIF-1 α stabilizer and SOX2 has been reported to interact with the HIF-1 α promoter (23, 24). Our results confirmed that treating AGS SORE6[−] cells for 48 h with 8 μ M CPX significantly increased the expression of HIF-1 α (Figure 5A). Since HIF-1 α expression is higher in CSCs and the AGS SORE6⁺ cells expressed significantly more HIF-1 α than AGS SORE6[−] cells (Figure 5A), we decided to explore whether this mechanism could be responsible for inducing the expression of SOX2 and consequently activating the SORE6-GFP reporter system. We started by stabilizing/increasing HIF-1 α expression using CoCl₂, a hypoxia-mimetic agent capable of increasing the expression of stem cell markers (25, 26). To achieve this, we treated AGS SORE6[−] and Kato III SORE6[−] cells with different CoCl₂ concentrations for 48 h. Activation of the SORE6-GFP system was observed in both cell lines after treatment with 200 and 300 μ M CoCl₂ (approximately 21% and 19%, respectively, in AGS SORE6[−] cells, and 2% and 7%, respectively, in Kato III SORE6[−] cells) (Figure 5B,C). In addition, an increase in SOX2, C-MYC, and HIF-1 α expression was observed in both cell lines (Figure 5D,E). Although not as cytotoxic as CPX, CoCl₂ had dose-dependent cytotoxicity in AGS SORE6[−] cells, whereas Kato III SORE6[−] cells were more resistant to treatment (Figure 5F). To rule out the possibility that the observed GFP expression after CoCl₂ treatment was autofluorescence, we incubated AGS wt cells with 200 μ M CoCl₂ for 48 h, and no fluorescence was observed (Figure S1B). Additionally, cells treated with 100, 200, and 300 μ M CoCl₂ showed CPX-like changes in OCR, ECAR, ATP production and rate, and percentage of glycolysis (Figure S3A–E). CoCl₂ treatment significantly decreased the rate of mitochondrial OXPHOS ATP production as well as the ratio of mitochondrial to glycolytic ATP production (Figure S3C,D). The rate of glycolytic ATP production increased in cells treated with 200 μ M CoCl₂ for 48 h (Figure S3C). Overall, our results showed similar effects of CPX and CoCl₂. Furthermore, CoCl₂ also induced cellular senescence with consequent loss in cell proliferation (Figure S3F,G). Because CPX and CoCl₂ increased the expression of SOX2, C-MYC, and HIF-1 α (Figures 2G and 5A,D,E), we explored whether these compounds also increased the expression of other relevant stemness markers in SORE6⁺ and SORE6[−] cells. Real-time PCR results revealed that GATA6, RELA, STAT3, KLF4, OCT1, and HMGA1 were significantly upregulated in AGS SORE6⁺ cells. In particular, HMGA1 was expressed more than 2-fold in AGS SORE6⁺ cells than in AGS SORE6[−] cells (Figure S4). In Kato III SORE6⁺ cells, C-MYC, HMGA1, and KLF4 were significantly upregulated. Specifically, KLF4 was expressed more than 6-fold in Kato III SORE6⁺ cells than in Kato III SORE6[−] cells (Figure S4). Notably, AGS SORE6[−] and Kato III SORE6[−] cells treatment with 8 μ M CPX (for 48 h) also significantly increased the expression of HMGA1, C-MYC, and HIF-1 α (Figure 5G). In AGS SORE6[−] cells, CPX treatment also increased KLF4 expression (Figure 5G). Similarly, 200 μ M CoCl₂ treatment significantly increased C-MYC, HIF-1 α , HMGA1, KLF4, and NANOG expression in AGS SORE6[−] cells (Figure 5G). In Kato III SORE6[−] cells, 300 μ M CoCl₂ upregulated C-MYC, HIF-1 α , KLF4, NANOG, and OCT4 (Figure 5G).

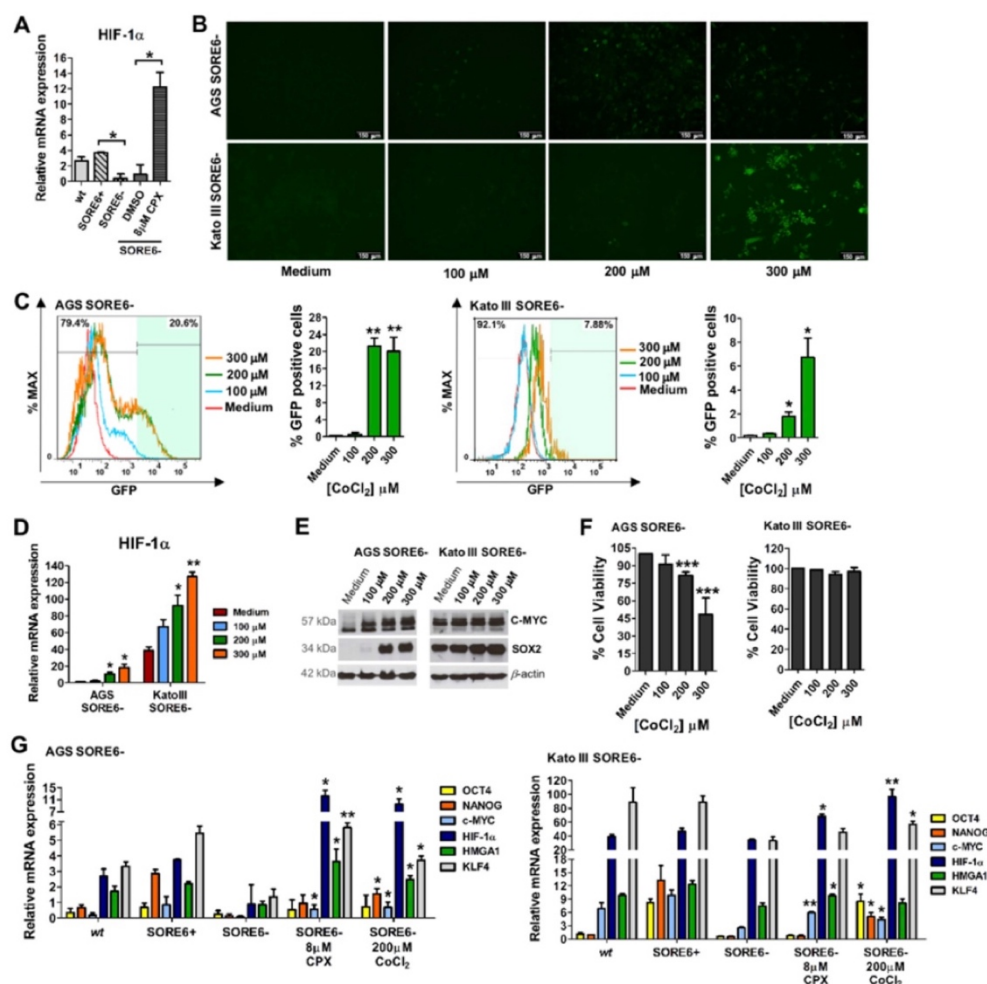


Figure 5. CPX and CoCl₂ treatment can effectively activate the SORE6–GFP reporter system and increase the expression of several transcription factors associated with stemness. (A) Real-time PCR analysis exhibiting the mRNA levels of HIF-1α in AGS wt, AGS SORE6+, AGS SORE6– and AGS SORE6– cells after 48 h treatment with DMSO or 8 μM CPX. (B) Representative fluorescence images of SORE6–GFP reporter system activation, scale bar = 150 μm, and (C) percentage of GFP-positive cells in AGS SORE6– and Kato III SORE6– cells treated with 100, 200, or 300 μM CoCl₂ for 48 h, following the respective flow cytometry analysis. (D) Real-time PCR analysis exhibiting the mRNA levels of HIF-1α in AGS SORE6– and Kato III SORE6– cells after treatment with 100, 200, or 300 μM CoCl₂ for 48 h. (E) SOX2 and C-MYC expression, evaluated by Western blot and (F) a percentage of cell viability after treatment of AGS SORE6– and Kato III SORE6– cells with 100, 200, or 300 μM CoCl₂ for 48 h; pictures show cropped areas of Western blots, the whole images are included in the Supplementary Materials (G) Real-time PCR analysis of mRNA levels of the transcription factors OCT4, NANOG, C-MYC, HIF-1α, HMGA1, and KLF4 in wt, SORE6+, SORE6–, and SORE6– cells treated with 8 μM CPX or 200 μM CoCl₂ for 48 h. DMSO or medium was used as a negative control. wt corresponds to the parental cell line (AGS or Kato III). Real-time PCR results were normalized to 18S expression. β-actin was used as a loading control. Results are mean ± SD of at least three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4. Discussion

CSCs constitute a very small population of cells present in the tumor. Evidence from experimental models and clinical studies indicates that CSCs are responsible for therapy resistance and disease recurrence [32–34]. Therefore, studying their characteristics is essential to understand how to target them. Since this is a very small population, several strategies have been considered to increase cell number in order to perform functional assays. One of these strategies is cellular reprogramming, which refers to the process of reverting specialized cells into induced pluripotent stem cells by rewiring transcriptional and epigenetic networks. During cellular reprogramming, the cell behavior, DNA methylation, histone modifications, and gene expression profiles can undergo several modifications [35–37]. In cancer, cellular reprogramming is an interesting approach to convert malignant cells into benign cells, but also to reprogram cancer cells into CSCs in order to generate a greater CSC population to evaluate their biological properties to improve cancer diagnosis and treatment [12,13,38].

Here, we used our previously established and characterized CSC model [7], which is based on SOX2 and OCT4 activity (SORE6+ (CSCs) vs. SORE6– (non-CSCs)), as a robust platform for the screening of small molecules that can impose CSC properties on SORE6– cells. Activation of the SORE6–GFP reporter system was used as a readout for successful cellular reprogramming. Our screening approach identified CPX, a broad-spectrum synthetic antifungal agent with antibacterial and anti-inflammatory activities [39], as an activator of the reporter system in SORE6– cells. Since SORE6– cells do not become GFP+ over time when they are treated with DMSO or medium, we are confident that CPX is inducing cellular reprogramming. Furthermore, we confirmed that this activation was associated with increased SOX2 expression. CPX also displayed a potent cytotoxic effect on the tested gastric cancer cells, decreasing cell viability and colony formation. Since CPX has been shown to have anti-tumorigenic properties and repress cell growth, migration, and invasion [40–45], its repurposing for cancer treatment has been already proposed [46]. Clinical studies have been performed on patients to assess CPX efficiency in the cancer setting [46,47]. However, despite the anti-tumor effect of CPX, we also observed that cells can easily acquire resistance to CPX treatment, which can be problematic for the use of this compound as a chemotherapeutic agent.

CPX has been reported to be associated with OXPHOS inhibition, and it also showed pro-senescent activity, linked to its activity as an iron (Fe^{3+} and Fe^{2+}) chelator [27]. Our results fully corroborate the ability of CPX to decrease OXPHOS and induce cellular senescence. Although this induction of cellular senescence may represent a blockage in tumor growth, it may also have the opposite effect of increasing tumor progression and resistance to therapy. This could be related to the close link between cellular senescence and the emergence of CSCs [48]. Accordingly, our results support the idea that senescence can be overcome, and cells can regain proliferative activity. Senescence can be induced by chemotherapeutic agents (e.g., cisplatin and doxorubicin) and radiotherapy [49,50]. Senescent cells acquire a senescence-associated secretory phenotype that can lead to the senescence-associated reprogramming of non-CSCs towards a stem-like phenotype [9,51,52]. It is still in debate if the senescent cells are the ones that reprogram or if they create an environment prone to cellular reprogramming via the secretion of factors that induce reprogramming in neighboring cells, which are known as Senescence Associated Secretory Proteins (SASPs). Recently, Lu and Zhang [49] reported that radiochemotherapy triggers DNA damage response in gastric cancer cells reprogramming them into gastric cancer stem-like cells via the EID3-NAMPT-Wnt/ β -catenin axis. They also showed that treatment with doxorubicin or cisplatin increased the expression of stemness markers ALDH1, OCT3/4, SOX2, C-MYC, and KLF4 in gastric non-CSCs [49]. Our results also indicated that treatment of gastric non-CSCs with CPX upregulated the expression of several stemness markers, including SOX2, C-MYC, KLF4, HMGA1, and HIF-1 α . Moreover, we postulate that the mechanism of cellular reprogramming exhibited by CPX may be similar to that of CoCl_2 , a well-known hypoxia-mimetic agent that stabilizes HIF-1 α expression by

blocking its degradation [53]. CoCl₂ treatment promotes the expression of stem cell markers such as SOX2, OCT4, C-MYC, and NANOG in a dose-dependent manner [54,55]. Our results confirmed that CoCl₂ treatment increased the expression of SOX2, C-MYC, KLF4, OCT4, NANOG, and HMGA1, showing similarities with the effect of CPX in promoting CSCs by upregulating the same set of TFs. Lopez-Sánchez et al. reported that treatment of colon cancer cells with CoCl₂ gave rise to polyploid giant cells (larger cells and greater nuclear size) with cancer stem-like features [56]. Interestingly, we also observed an increase in cell size after CPX and CoCl₂ treatments in the two gastric cancer cell lines we have studied. Similarly to CPX, CoCl₂ treatment also demonstrated pro-senescent activity and promoted a metabolic shift towards glycolysis, reducing OXPHOS. Metabolically, CSCs can change the way they obtain ATP, depending on the type of tumor and the conditions of the tumor microenvironment. For example, in hypoxic environments, CSCs appear to prefer producing ATP via glycolysis rather than OXPHOS, even in the presence of oxygen (Warburg effect) [57–59]. With hypoxia being a common feature of the microenvironment of solid tumors, it is worth mentioning that hypoxia and its master regulators—hypoxia-inducible factors (HIFs)—participate in stem-like maintenance in various cancers and dysregulate stemness genes such as SOX2 [60,61].

5. Conclusions

Together, our results, although supporting the powerful cytotoxic effect of CPX on tumor cells, also recommend care if its therapeutic use is intended, since it may kill most tumor cells but may also convert surviving cells into CSCs. At least at certain concentrations and exposure times, the potential for cellular reprogramming is present via induction of cellular senescence (which can be reversed), metabolic plasticity, and increased expression of stemness markers (e.g., SOX2, C-MYC, KLF4, HMGA1). Therefore, CPX may be a useful tool for obtaining gastric cancer stem-like cells from non-CSCs, particularly when cells previously treated with CPX are re-exposed to the compound. On the other hand, in an anti-tumor context, to potentiate its cytotoxic effect and avoid cellular reprogramming of gastric non-CSCs towards a stem-like state, adjustments in CPX concentration and exposure period should be evaluated.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cancers15174406/s1>, Table S1: Primers used for real-time PCR analysis. Figure S1: Evaluation of the percentage of GFP-positive cells by flow cytometry. Figure S2: CPX-induced cellular senescence is reversible and cells regain the proliferative ability. Figure S3: CoCl₂ treatment inhibits ATP production by OXPHOS and induces cellular senescence with consequent loss of proliferative activity. Figure S4: Comparison of the expression of relevant transcription factors between SORE6+ and SORE6− cells. Uncropped Western blot images corresponding to all the figures in the manuscript.

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

Funding: This research was funded by the project Norte-01-0145-FEDER-000051-“Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets”, supported by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, via the European Regional Development Fund (FEDER). Diana Pádua acknowledges Fundação para a Ciência e a Tecnologia (FCT) for financial support via a Ph.D. fellowship (SFRH/BD/146186/2019), co-sponsored by Fundo Social Europeu (FSE) via the North Portugal Regional Operational Programme. Patrícia Mesquita acknowledges financial support via Programa Operacional Regional do Norte and European Regional Development Fund under the project “The Porto Comprehensive Cancer Center” with the reference NORTE-01-0145-FEDER-072678—Consórcio PORTO.CCC—Porto.Comprehensive Cancer Center.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data obtained and analyzed during this work are presented in the published article together with its Supplementary Materials. The data are also available from the authors upon request.

Acknowledgments: The authors acknowledge the support of the i3S Scientific Platform BioSciences Screening Advanced Light Microscopy, member of the national infrastructures PT-OPENSCREEN (NORTE-01-0145-FEDER-085468) and PPBI—Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122). The authors also acknowledge the support of the Cell Culture and Genotyping, Genomics, and Biochemical and Biophysical Technologies i3S Scientific Platforms. We thank Mafalda Sousa for having developed the custom MATLAB script.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

High-Throughput Drug Screening Revealed That Ciclopirox Olamine Can Engender Gastric Cancer Stem-like Cells

Table S1: Primers used for real-time PCR analysis

Gene	Primer Forward (5' → 3')	Primer Reverse (5' → 3')
ARNT2	AAGAACCGGGAGTGGATGTTG	CTGTTGCTGAAGTTGCTTGACG
β-catenin	CAATGGCTTGAATGAGACTGC	GGATCATCCTGGTGATATCCAAG
c-Jun	AGTGAGTGACCGCGACTTTT	GATGCCTCCCGCACTCTTAC
C-MYC	GCGACTCTGAGGAGGAACAAG	TGGGCTGTGAGGAGGTTTG
CNOT3	GGCTCACGAATACCATCGACA	GCTTATCCTTGTGCGCCCTTCT
FOXA1	AAGGGCATGAAACCAGCGAC	GCCTGAGTTCATGTTGCTGAC
FOXA2	ATGCACTCGGCTTCCAGTAT	GTTGCTCACGGAGGAGTAGC
FOXC2	CTACAGCTACATCGCGCTCATCA	ACTGGTAGATGCCGTTCAAGGTG
GATA6	AAGCGCGTGCCTTCATCA	CATAGCAAGTGGTCTGGGCA
GLI1	GAAGTCATACTCACGCCTCGAA	CAGCCAGGGAGCTTACATACAT
GLI2	AGCAGCAGCAACTGTCTGAGTGA	GACCTTGCTGCGCTGTGAA
Hes1	ACACGACACCGGATAAACCA	ATGCCGCGAGCTATCTTTCT
HIF-1α	GGCGGAACGACAAGAAAAAGATA	GGCCTTATCAAGATGCGAACTCAC
HMGA1	GTGCCAACACCTAAGAGACCT	TCTGCTGGTTTCCGGCTC
HMGA2	CCCAAAGGCAGCAAAAACAA	GCCTCTTGGCCGTTTTTCTC
HOXA10	CCCTTCCGAGAGCAGCAAA	TCTTCCGACCACTCTTTGCC
HOXA5	GCGCAAGCTGCACATAAGTC	CGGAGAGGCAAAGAGCATGT
ISL1	CTGCTTTTCAGCAACTGGTCA	TAGGACTGGCTACCATGCTGT
KLF4	CAGAGGAGCCCAAGCCAAAG	TTTCTCACCTGTGTGGGTTCG
KLF5	AAGGAGTAACCCGATTG	CAGCCTTCCAGGTACACTT
MEIS2	TCCAGCATCTCACACATCCG	GAAAACCTGCTCGATTGACTGG
NANOG	GAACCTCAGTACAAACAGGTGAA	TTCTGCGTCACACCATTGCT
OCT1	CAAAATGGCGGACGGAGGA	GTTTATTCTTGAGTCTGCTGCTG
OCT4	TGCAGCAGATCAGCCACAT	ACACTGGTCCCCCTGAGAAA
RelA	GCTGCATCCACAGTTTCCAGA	CCCCACGCTGCTTTCTAT
SOX4	GACTTCGAGTTTGCTCCCT	TAACCTCGCTTCTTGCTGGG
SOX9	CGGAGGAAGTCGGTGAAG	CTGGGATTGCCCGAGTGCT

STAT3	GAGGACTGAGCATCGAGCA	CATGTGATCTGACACCCTGAA
TCF4	TTTGGAAGAAGCGCCAAGAGG	TTGGGAGGTAGGGGCTCTGT
TEAD2	TTTGGGGTGTGCCCAGATG	TCCTCACTGCCTTCCTCACT
ZNF273	AGCCTAGAAATGGGACCACCTG	GCTGTGAAGTGCCAGGCAT
18S	CGCCGCTAGAGGTGAAATTC	CATTCTTGGCAAATGCTTTCG

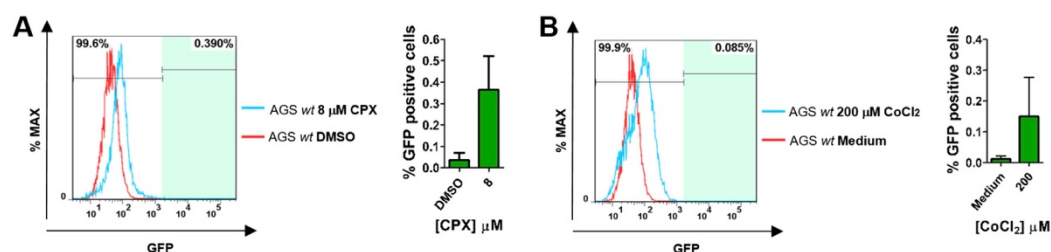


Figure S1: Evaluation of the percentage of GFP positive cells by flow cytometry. Flow cytometry analysis of GFP expression in AGS wt cells **(A)** after 48 h of treatment with 8 μ M CPX and **(B)** after 48 h of treatment with 200 μ M CoCl₂. wt corresponds to the parental cell line (AGS). DMSO or medium were used as a negative control. Results are mean $\pm$ SD of at least three independent experiments.

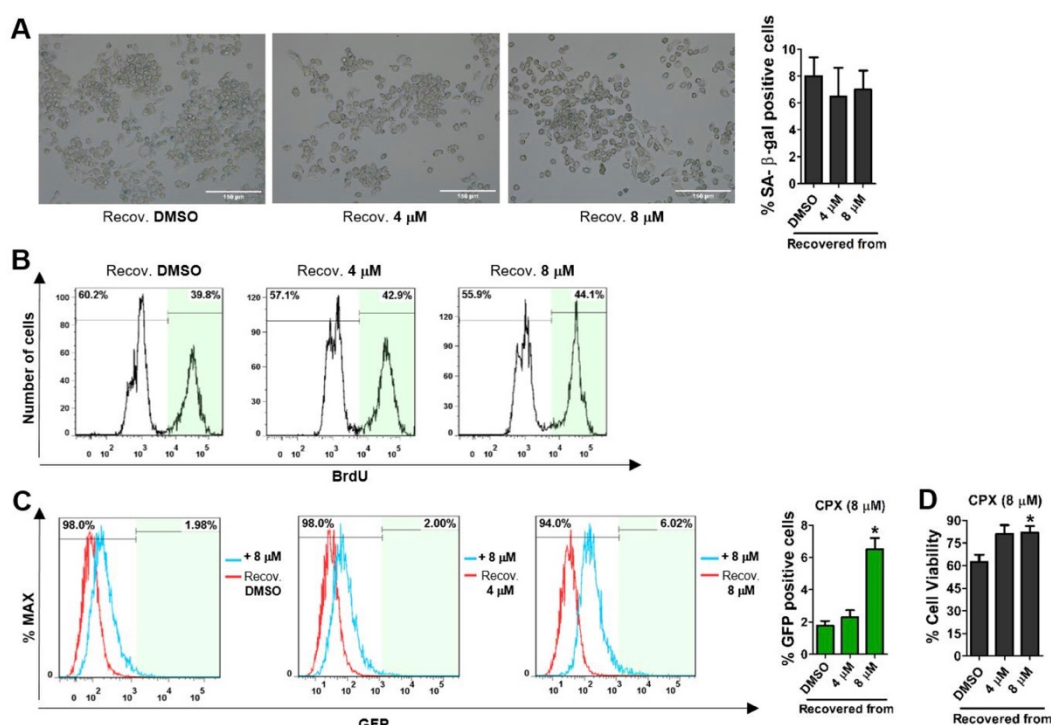


Figure S2: CPX-induced cellular senescence is reversible and cells regain the proliferative ability. (A) Percentage of SA- β -gal positive cells in Kato III SORE6- cells 4 weeks after recovery from treatment with DMSO, 4 or 8 μ M CPX for 48 h, following representative images of SA- β -gal marking. Scale bar = 150 μ m. (B) Representative flow cytometry analysis of the percentage of BrdU positive cells in AGS SORE6- cells 4 weeks after recovery from treatment with DMSO, 4 or 8 μ M CPX for 48 h. (C) Percentage of cell viability after 48 h of treatment of AGS SORE6- and Kato III SORE6- recovered cells with 4.5 μ g/mL (IC50) of 5-FU. (D) Percentage of GFP-positive cells, following the respective flow cytometry analysis, and (E) cell viability in Kato III SORE6- recovered cells after new treatment with 8 μ M CPX for 48 h. DMSO was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. Significant differences * $p \leq 0.05$.

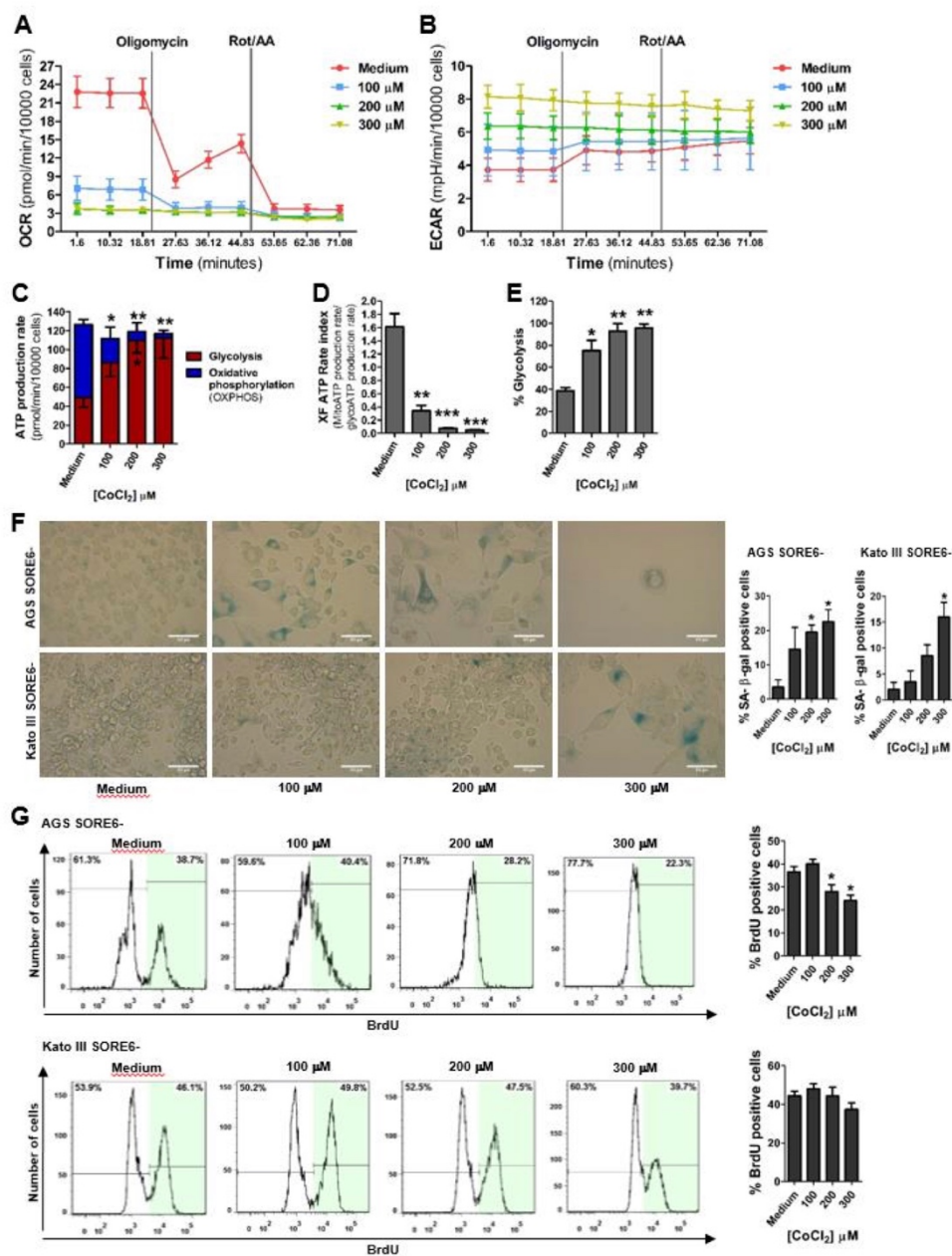


Figure S3: CoCl₂ treatment inhibits ATP production by OXPHOS and induces cellular senescence with consequent loss of proliferative activity. (A) Representative real-time measurements of the kinetic profile of the OCR, (B) representative real-time measurements of the kinetic profile of the ECAR, (C) metabolic flux analysis showing quantification of mitochondrial ATP production and glycolytic ATP production, (D) ATP production rate index calculated from the data showed in H (i.e., mitochondrial ATP production rate/glycolytic ATP production rate) and (E) percentage of glycolysis following treatment with 100, 200 or 300 μ M CoCl₂ for 48 h. (F) Percentage of SA- β -gal positive cells in AGS SORE6- and Kato III SORE6- cells treated with 100, 200 or 300 μ M CoCl₂ for 48 h, followed by representative images of SA- β -gal marking. Scale bar = 150 μ m. (G) Percentage of

BrdU positive cells in AGS SORE6⁺ and Kato III SORE6⁺ cells treated with 100, 200 or 300 μ M CoCl₂ for 48 h, followed by representative flow cytometry analysis. Medium was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

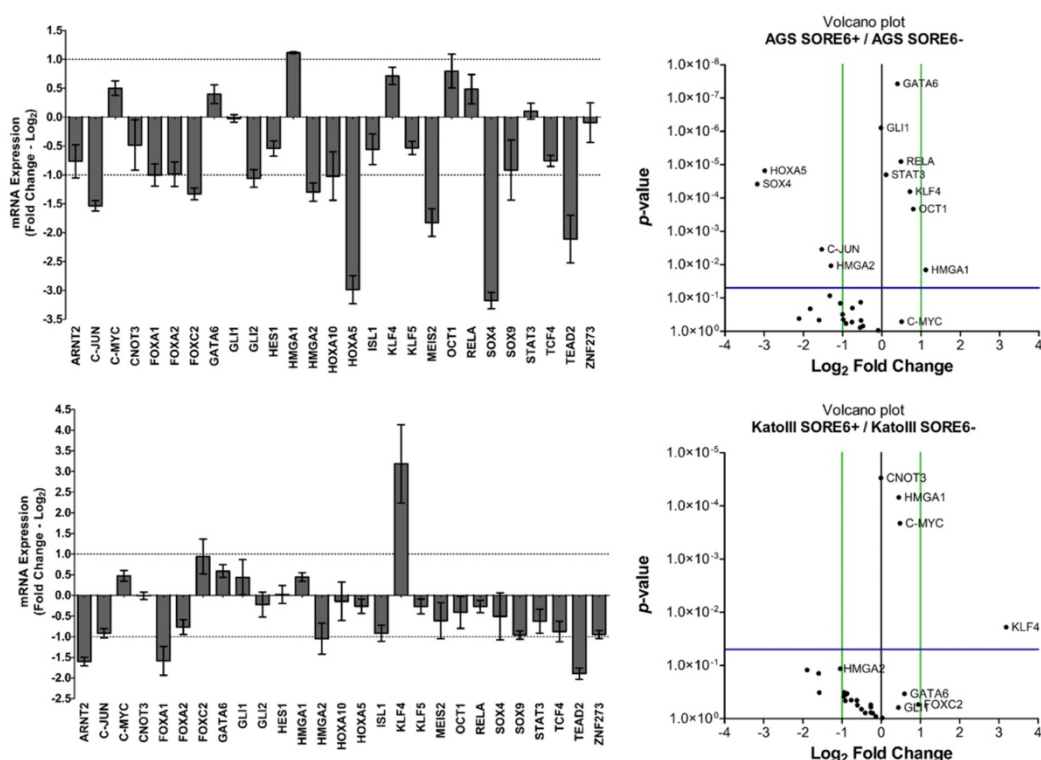
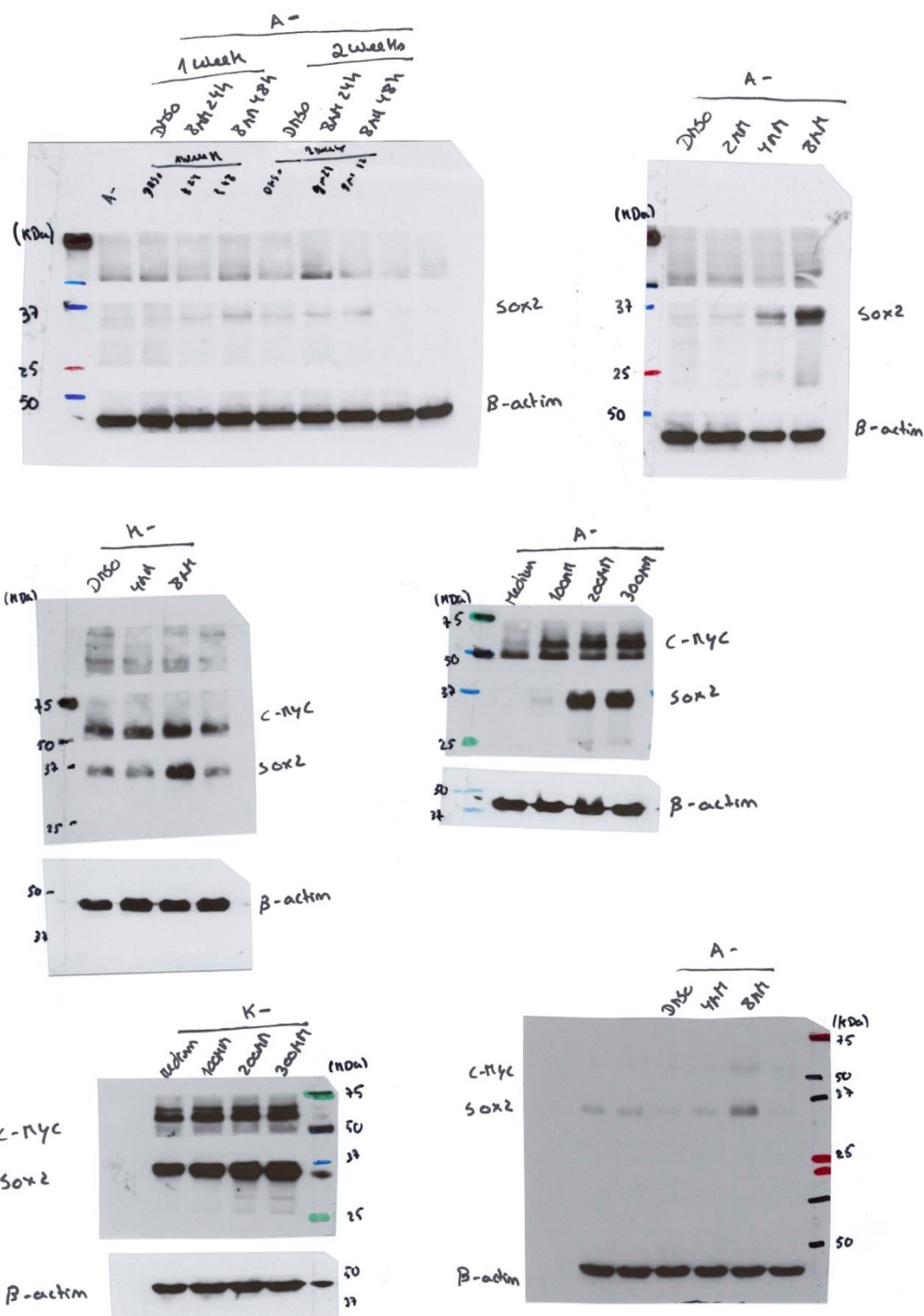


Figure S4: Comparison of the expression of relevant transcription factors between SORE6⁺ and SORE6⁻ cells. Real-time PCR analysis of mRNA levels of the transcription factors ARNT2, C-JUN, C-MYC, CNOT3, FOXA1, FOXA2, FOXC2, GATA6, GLI1, GLI2, HES1, HMGA1, HMGA2, HOXA10, HOXA5, ISL1, KLF4, KLF5, MEIS2, OCT1, RELA, SOX4, SOX9, STAT3, TCF4, TEAD2 and ZNF273 in AGS SORE6⁺ and Kato III SORE6⁺ cells in comparison to AGS SORE6⁻ and Kato III SORE6⁻ cells. Followed by the volcano plot (SORE6⁺ compared to SORE6⁻ cells) where the horizontal blue line represents the threshold of statistical significance ($p = 0.05$) and the green lines corresponds to the fold change cut-off ≥ 2 . Results were normalized to 18S expression. Results are mean $\pm$ SD of at least three independent experiments.

UNCROPPED WESTERN BLOT FIGURES



CHAPTER IV

UPREGULATION OF HMGA1 CONFERS CANCER STEM-LIKE FEATURES TO GASTRIC NON-CSCS

This chapter contains the following work:

Pádua, D., Figueira, P., Pombinho, A., Monteiro, I., Pereira, C. F., Almeida, R., & Mesquita, P. *Upregulation of HMGA1 confers cancer stem-like features to gastric non-CSCs. In Preparation*

In Chapter III, we showed that we were able to reprogram gastric non-CSCs into cancer stem-like cells by treating SORE6- cells with the antifungal agent CPX. Depending on the dose and exposure time, CPX treatment was able to activate the reporter system by inducing/increasing SOX2 expression. It also increased the expression of C-MYC, HIF-1 α , KLF4 and HMGA1. As mentioned in Chapter I, HMGA1 expression positively regulates SOX2 and OCT4 expression and has been reported as a key player in cellular reprogramming. Its addition to the Yamanaka TFs cocktail increases the efficiency of cellular reprogramming. Interestingly, HMGA1 is an essential component of the chromatin architecture of senescent cells and regulates glycolysis in gastric cancer. Furthermore, HMGA1 has been linked to drug resistance. So, we decided to explore the role of HMGA1 expression on the characteristics of gastric CSCs. In this way, we attempted to reprogram non-CSCs into CSCs through TF-mediated cellular reprogramming. Therefore, again using the SORE6-GFP reporter system incorporated into SORE6- cells, as a readout of successful cellular reprogramming, we transduced SORE6- cells, from two gastric cancer cell lines (AGS and SNU638), with a HMGA1 doxycycline (DOX)-inducible lentiviral vector system.

In this Chapter, *Upregulation of HMGA1 confers cancer stem-like features to gastric non-CSCs*, we show that HMGA1 was significantly more expressed in gastric cancer tissue compared to normal gastric tissues. Overexpression of HMGA1 in SORE6- cells led to the expression of SOX2 which consequently activated the SORE6-GFP reporter system. Moreover, SORE6- cells transduced with the HMGA1 doxycycline (DOX)-inducible lentiviral vector were sorted into two cell population, HMGA1 GFP- and HMGA1 GFP+ cells, based on GFP expression. Unlike HMGA1 GFP- cells, HMGA1 GFP+ cells were enriched in SOX2, C-MYC and KLF4. The HMGA1 GFP+ cells showed CSCs features, such as asymmetric division, greater ability to proliferate and form colonies, and resistance to 5-FU compared to HMGA1 GFP- cells. Additionally, we validated that CPX treatment induces HMGA1 expression in a dose-dependent manner. We also verified that overexpression of HMGA1 increases the cellular reprogramming efficiency of non-CSCs into cancer stem-like cells in the presence of CPX. Our data also indicated that, like SORE6+ cells (CSCs), HMGA1 GFP+ cells are sensitive to monensin. In particular, the GFP+ cells in the HMGA1 GFP+ cell population appeared to be the preferential target of monensin treatment. Overall, our results suggest that SOX2 could be at the top of the hierarchy in gastric CSC transcription factors network and that HMGA1 plays an essential role in the induction/maintenance of the gastric CSC phenotype.

Upregulation of HMGA1 confers cancer stem-like features to gastric non-CSCs

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Abstract

Gastric cancer represents a serious health problem worldwide, with insufficient diagnostic markers/methods and therapeutic options. However, several efforts have been directed towards finding specific disease markers in order to develop new therapies capable of defeating gastric cancer. Attention has been pointed to cancer stem cells (CSCs) as they are primarily responsible for tumor initiation and recurrence, making them essential therapeutic targets. Using the reported SORE6-GFP system, based on the expression of SOX2 and/or OCT4 to drive GFP expression, we isolated gastric cancer stem-like cells (SORE6+ cells) enriched in several molecules, including SOX2, C-MYC, KLF4, HIF-1 α , NOTCH1 and HMGA1, and also non-CSCs (SORE6- cells). The transcription factor HMGA1 is of particular interest because it is associated with promoting a cancer stem-like phenotype. Thus, we explored the impact of overexpressing HMGA1 on gastric non-CSCs. Our results indicated that HMGA1 can activate the SORE6-GFP system embedded in SORE6- cells, by upregulating SOX2 expression. Furthermore, we sorted the cells into HMGA1 GFP+ and HMGA1 GFP- cell populations and found that HMGA1 GFP+ are enriched in SOX2, C-MYC, and KLF4, had characteristics of CSCs, and were sensitive to

monensin just like SORE6+ cells. We also validate that HMGA1 expression enhances cellular reprogramming of gastric non-CSCs to cancer stem-like cells.

Keywords: Gastric Cancer, Cancer Stem Cells, SOX2, Transcription Factors, Drug Resistance, Monensin, Cellular Reprogramming,

1. Introduction

Gastric cancer continues to represent a serious health problem worldwide. It is the fifth most diagnosed cancer and the fourth deadliest (1). The success of gastric cancer treatment depends mainly on the stage at which the disease is diagnosed. But as it is a silent disease in the early stages of development, most patients are only diagnosed in more advanced stages, where the best treatment options can no longer be used and, therefore, the prognosis is poor (2, 3). To worsen this problem, the presence of CSCs in tumors maintains tumor mass through self-renewal and asymmetric division, promotes resistance to radio and chemotherapy and also promotes the spread of cancer to other parts of the body through metastases (4-6). Therefore, it is important to discover new forms or biomarkers that allow for an earlier identification of this pathology and identification of CSCs, so that target therapies can be developed and/or used in order to eliminate one of the major obstacles in cancer treatment (7-9). Various biomarkers have been used to identify CSCs, especially cell-surface markers. CD44 was the first cell-surface marker used to identify gastric CSCs (10). However, the use of transcription factors proved to be more robust. These are proteins that bind to specific DNA sequences and control gene expression in response to different stimuli or growth conditions (11). Several transcription factors have been associated with the induction and maintenance of the stem-like characteristics exhibited by embryonic stem cells and CSCs. SOX2, OCT4, KLF4, C-MYC and NANOG expression has been reported to maintain the signature features of stem-like cells, but they are not the only ones (12). For instance, overexpression of SOX2, OCT3/4, KLF4 and C-MYC generated iPS cells from non-stem cells (13, 14). And the addition of the HMGA1 transcription factor to this pool increases the reprogramming efficiency of iPS cells (15). Therefore, HMGA1 has sparked some interest in stemness. HMGA1 is a chromatin architectural protein that interacts with the transcriptional machinery to negatively or positively regulate the transcription of several genes (16). Shah et al. showed that knockdown of HMGA1 leads to downregulation of SOX2, OCT4, C-MYC and LIN28, possibly by direct interaction with their promoters (15). This transcription factor is highly expressed in several cancers and has been associated with aggressiveness of the tumor,

occurrence of metastasis and poor survival (17-20). In gastric cancer, HMGA1 promotes tumor growth, metastasis and regulates aerobic glycolysis (21-23). Additionally, several studies have linked the high levels of HMGA1 expression to the CSC phenotype (24-26).

We used the SORE6-GFP reporter system to isolate and characterize two gastric cancer cell populations based on GFP expression (SORE6⁻ and SORE6⁺ cells) (28). SORE6⁺ cells were considered gastric cancer stem-like cells while SORE6⁻ cells were considered as non-CSCs. SORE6⁺ cells were enriched in SOX2, C-MYC, NOTCH1, KLF4, HIF-1 α and HMGA1 (28, 29). Our results also showed that SOX2 expression plays a crucial role in 5-FU resistance (28). Additionally, we managed to find a small molecule, monensin (a polyether ionophore antibiotic), which appears to selectively eliminate SORE6⁺ cells (28). Recently, we also showed that gastric CSCs can be generated by ciclopirox olamine (CPX) treatment of SORE6⁻ cells (29). CPX treatment induced/increased SOX2, C-MYC, KLF4, HIF-1 α and HMGA1 (29).

Little is known about HMGA1 expression in gastric CSCs. Therefore, in this work, we explored the particular role of HMGA1 in the stemness properties of gastric CSCs and its influence on SOX2 expression. We also investigated the association between HMGA1 expression and CPX treatment and its relevance in CPX-mediated cellular reprogramming of gastric non-CSCs into cancer stem-like cells.

2. Materials and Methods

2.1. Cell Culture

Two human gastric carcinoma cell lines (AGS SORE6⁻ and AGS SORE6⁺) established under the scope of a previous work (28) were used in this study. SNU SORE6⁻ and SNU SORE6⁺ gastric cancer cells were established as described in (27, 28). Furthermore, the human gastric carcinoma cell lines AGS (ATCC, CRL-1739, Manassas, VA, USA) and SNU638 (from IPATIMUP's cell line bank), from which the SORE6 cell lines were derived, were also used, as well as the human embryonic kidney cell line, HEK293T (kindly provided by Dr. João Relvas from Glial Cell Biology Group, i3S, Porto, Portugal). The gastric cancer cell lines were cultured in RPMI medium 1640 with 25 mM Hepes and GlutaMAX-1 (Gibco, Life Technologies, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France). HEK293T cells were cultured in DMEM with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Life Technologies, Thermo Fisher) supplemented with 10% FBS. All cells were maintained at 37 °C and 5% (v/v) CO₂. When the cell cultures reached a confluence around 80% they were trypsinized with 0.05%

Trypsin-EDTA (1X) (Gibco, Life Technologies, Thermo Fisher) and sub-cultured or used in cell-based assays.

2.2. Viral Supernatant Production and Cell Transduction

The doxycycline (DOX)-inducible lentiviral vectors pFUW-M2rtTA (M2rtTA) and pFUW-tetO-HMGA1 (HMGA1) were constructed by Dr. Carlos Filipe Pereira team at CNC/UC-BIOTECH, Coimbra, Portugal. The lentivirus particles were generated using HEK293T cells as packaging cell line. The cells were seeded in 10 cm dishes at a density of 1×10^6 cells/mL and incubated for 24 h. Then, each lentiviral vector was co-transfected with the psPAX2 packaging plasmid and the pVSV-G envelope plasmid into HEK293T cells using Lipofectamine 2000 (Invitrogen, Waltham, MA, EUA) in a 1 µg DNA: 1.5 µL lipofectamine ratio in serum- and antibiotic-free Opti-MEM medium (Gibco, Life Technologies, Thermo Fisher). After 24 h of incubation, medium was changed to DMEM medium without phenol red (1X) with 4.5 g/L D-Glucose, L-Glutamine and 25 mM Hepes (Gibco, Life Technologies, Thermo Fisher) supplemented with 10% FBS and 1% Sodium Pyruvate (Gibco, Life Technologies, Thermo Fisher) and cells were left undisturbed for 48 h. Viral supernatants were centrifuged at 3000 rpm for 15 min at RT and passed through a 0.22 µm pore PES filter (Orange Scientific, Braine-l'Alleud, Belgium) and used right away or stored at -80 °C. AGS SORE6- cells and SNU SORE6- cells were seeded in 6-well plates at a density of 1.5×10^5 cells/mL and allowed to settle for 24 h. Cells were transduced with the HMGA1 doxycycline (DOX)-inducible lentiviral vector, diluted 1:3 in a mixture containing DMEM complete medium without phenol red/M2rtTA/HMGA1 for 48 h. Cells transduced only with M2rtTA were used as negative control. Following transduction, fresh medium supplemented with DOX (1 µg/ml) was added for at least 48 h prior to any assay. Selection with 400 µg/ml of zeocin was performed to ensure that all cells were transduced.

2.3. Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS)

Cells were collected by trypsinization and centrifuged at 1200 rpm for 5 min at RT. Cell pellets were washed three times with PBS (1X) prior to resuspension in PBS (1X) containing 10% FBS. Flow cytometry analysis was performed on FACSCanto II flow cytometer (BD Biosciences; Franklin Lakes, NJ, USA). Cells transduced with pFUW-tetO-HMGA1 were sorted using a FACSaria II Cell Sorter flow cytometer (BD Bioscience), cells transduced with FUW-M2rtTA viral supernatant were used as negative control for gating assessment. Cells with and without GFP signal were sorted out and seeded in 6-well plates at a density of 5×10^4 cells/mL for further experiments. Sorted cells were cultured in RPMI medium

supplemented with 20% FBS and 1% penicillin-streptomycin and maintained at 37 °C and 5% (v/v) CO₂ in a humidified incubator. The data was analyzed using FlowJo software (version 7.6.1).

2.4. Protein Extraction and Western Blot

Whole cell extracts were prepared as described in (28). After, 30 µg of total protein extracts were separated by standard SDS-PAGE using the Precision Plus Protein Standard Dual Color protein marker (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Amersham, GE Healthcare, Chicago, IL, USA). Then, membranes were washed with TBS-1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and blocked with either 5% non-fat milk in TBS-1% Tween-20 or 5% BSA (Sigma-Aldrich) for 1 h at room temperature (RT). The membranes were incubated overnight at 4 °C with the following primary antibodies: SOX2 (diluted 1:500; Cell Marque, Sigma-Aldrich); C-MYC (diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA); HMGA1 (diluted 1:500; Abcam, Cambridge, UK; ab252930); and β-actin (diluted 1:4000; Santa Cruz Biotechnology, Dallas, TX, USA). Later, membranes were washed with TBS-1% Tween-20 and incubated for 1 h at RT with the respective HRP- conjugated secondary antibody: rabbit IgG (HRP) (diluted 1:10000; Cell signaling) or mouse IgG (HRP) (diluted 1:2000; Santa Cruz Biotechnology). Signal detection was performed using the ECL detection kit (Amersham, GE Healthcare). β-actin was used as loading control.

2.5. RNA Extraction and Real-Time PCR

Cells were collected by trypsinization and cell pellets were washed with PBS (1X). Total RNA was extracted from cell pellets using TRI Reagent (Sigma-Aldrich; T9424), according to the manufacturer's protocol. Concentration and quality of the RNA were assessed using NanoDrop ND-1000 spectrometer (V3.5.2 Software). Total RNA (1 µg) was converted to cDNA in a thermocycler (Bio-Rad) in a final reaction volume of 20 µL, containing 1 µL of random primers (100 ng/µL) (Invitrogen), 1 µL of dNTPs (10 mM) (Invitrogen), 2 µL of 10x Reaction Buffer (NZYTech, Lisboa, Portugal), 0.2 µL of RNaseOUT (40 U/µL) (Invitrogen), and 0.5 µL of NZY Reverse Transcriptase (200 U/µL) (NZYTech) in DEPC-treated water (Invitrogen). For the PCR, each reaction was prepared with 4 µL of cDNA diluted 1:20 in DEPC-treated water, 10 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.6 µL of each primer (10 µM), and 4.8 µL of DEPC-treated water (Invitrogen). The reactions were executed in a 7500 Fast Real-Time PCR System using the software v2.0.6 (Applied Biosystems) with the following thermal cycling conditions: 40

cycles of 15 seconds at 95 °C for denaturation, plus 1 min at 60 °C for annealing and a melting curve program (60-95 °C) with continuous fluorescence measurement. The primers used were the following: 18SFor: CGCCGCTAGAGGTGAAATTC; 18SRev: CATTCTTGGCAAATGCTTTTCG; OCT4For: TGCAGCAGATCAGCCACAT; OCT4Rev: ACACTGGTCCCCCTGAGAAA; HMGA1For: GTGCCAACACCTAAGAGACCT; HMGA1Rev: TCTGCTGGTTTTCCGGCTC; HMGA2For: CCCAAAGGCAGCAAAAACAA; HMGA2Rev: GCCTCTTGGCCGTTTTTCTC; NANOGFor: GAACCTCAGCTACAAACAGGTGAA; NANOGRev: TTCTGCGTCACACCATTGCT; KLF4For: CAGAGGAGCCCAAGCCAAAG; KLF4Rev: TTTCTCACCTGTGTGGGTTCG; C-MYCFor: GCGACTCTGAGGAGGAACAAG; C-MYCRev: TGGGCTGTGAGGAGGTTTG. Each reaction was performed in triplicate. Negative controls (without cDNA) were included in each plate and the 18S ribosomal RNA (rRNA) was used as a housekeeping gene. Data was analyzed using the comparative $2^{-\Delta\Delta CT}$ method (30).

2.6. Immunofluorescence

Cells were plated in 24-well plates, with one coverslip in each well, at a density of 1.5×10^4 cells/mL in RPMI medium supplemented with 10% FBS and allowed to attach overnight. When cells reached about 70%–80% confluence, medium was removed, cells were washed with PBS (1X), fixed with cold methanol (Chem-Lab, Zedelgem, Belgium) or paraformaldehyde (PFA) and washed again with PBS (1X). Then, the coverslips were washed with PBS (1X) and incubated with the primary antibody HMGA1 (diluted 1:500; Abcam) overnight at 4 °C. Next, coverslips were incubated with the secondary antibody goat anti-rabbit Alexa Fluor 488 (diluted 1:150; A11032, Thermo Fisher Scientific) for 45 min in the dark at RT. DAPI (dilution 1:100; Sigma-Aldrich) was added to the coverslips for nuclei staining. The coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and pictures were taken using a Carl Zeiss fluorescent microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

2.7. Clonogenic Assay

Cells were plated in 6-well plates at a density of 300 or 1000 cells per well and left undisturbed for 10 days at 37 °C and 5% (v/v) CO₂, except for the fifth day, when the medium was changed. After 10 days, colony formation was visualized by staining the cells with a 1% crystal violet solution diluted in PFA for 30 min. The number of colonies per condition was assessed with the ImageJ software.

2.8. 5-FU, Monensin and Ciclopirox Effects Assessment

Cells were seeded in 24-well plates or 6-well plates at a density of 0.7×10^4 or 0.6×10^5 cells/mL, respectively, in RPMI medium supplemented with 10% FBS. Twenty-four hours later, cells were treated with 4.5 $\mu\text{g/mL}$ of 5-FU, or 8 μM of monensin or CPX for 48 h. Cells treated only with DMSO (AppliChem GmbH, Darmstadt, Germany) were used as control. After 48 h, cells were collected by trypsinization and cell pellets were washed in PBS (1X) and resuspended in 400 μL of 1X Binding Buffer (Annexin V Apoptosis detection Kit APC, eBioscience, Thermo Fisher Scientific). Five μL of annexin V APC (Annexin V Apoptosis detection Kit APC, eBioscience, Thermo Fisher Scientific) were added to 100 μL of the cell suspension and incubated in the dark for 10 min at RT. Then, cells were washed and resuspended in 200 μL of 1X Binding Buffer. After, 5 μL of Propidium Iodide (PI) (Annexin V Apoptosis detection Kit APC, eBioscience, Thermo Fisher Scientific) were added to the cell suspension. The percentage of apoptotic cells was obtained using the FACSCanto II flow cytometer. The results were analyzed using FlowJo software (version 7.6.1); Cell viability was also assessed using PrestoBlue assay. For that, culture media was discarded, adherent cells were washed with PBS (1X), and 50 μL of PrestoBlue Cell Viability Reagent 1X (ThermoFisher Scientific) prepared in RPMI complete medium were added. Cells were incubated for 1 h and 10 min at 37 °C in a 5% CO₂ humidified atmosphere. Absorbance was measured at 560 nm (using 600 nm as reference wavelength) using a BioTek Synergy 2 multi-mode microplate reader. For further evaluation, cells were collected, and pellets washed. Cell pellets were used for flow cytometry analysis of GFP expression and for western blot or real-time PCR analysis of transcription factors expression. Cells treated with DMSO were used as negative control.

2.9. Cell Proliferation

Cells were cultured in 6-well plates at a density of 0.8×10^5 cells/mL in RPMI medium supplemented with 10% FBS. Twenty-four h later, BrdU was incorporated in the cell culture medium at the ratio of 1:1000, for 1 h at 37 °C. After, cells were harvested by trypsinization and cell pellets fixed in ice-cold methanol. The cells were resuspended in HCl 4 M (Mallinckrodt Baker, Avantor, Allentown, PA, USA) for 20 min at RT followed by two washing steps with PBS (1X) and a blocking step with PBS (1X) containing 0.5% Tween-20 and 0.05% BSA for 10 min. The cells were incubated with the primary antibody against BrdU (1:20, monoclonal mouse, Bu20a, Dako, Glostrup, Denmark) for 1 h at RT, washed with PBS (1X), and incubated with the secondary antibody labelled with FITC (1:150 μL , polyclonal rabbit anti-mouse, Dako) for 30 min, washed again and resuspended in PBS

(1X). The percentage of BrdU positive cells was assessed using the FACSCanto II flow cytometer. The data was analyzed using FlowJo software (version 7.6.1).

2.11. Statistical Analysis

Results were expressed as mean $\pm$ SD from at least three independent experiments. Statistical significance was determined by unpaired two-tailed t test or one-way ANOVA followed by post hoc Turkey's test using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. HMGA1 overexpression can activate the SORE6-GFP reporter system incorporated in SORE6- cells by inducing SOX2 expression

HMGA1 has been shown to play an important role in regulating and maintaining the stemness phenotype. Our previous work revealed that significantly elevated expression of HMGA1 was common to both AGS SORE6+ cells and AGS SORE6- cells treated with 8 μ M of CPX, both gastric cancer stem-like cells (29). To assess the expression of HMGA1 in gastric cancer, we used RNA sequencing data present in the Cancer Genome Atlas (TCGA) (31) and Genotype-Tissue Expression (GTEx) (32). Thus, we matched the expression of HMGA1 in 408 stomach adenocarcinoma (STAD) tissues with that of 211 normal tissues. The results indicated that HMGA1 is significantly more expressed in tumor tissues than in normal tissues (Figure 1A). Since HMGA1 was significantly higher in gastric cancer tissue, and in gastric cancer stem-like cells compared to non-CSCs (29), we decided to overexpress HMGA1 in SORE6- cells to investigate whether HMGA1 was able to activate the SORE6-GFP reporter system and to originate potential cancer stem-like cells. By overexpressing HMGA1, we detected a significant percentage of AGS SORE6- and SNU638 SORE6- cells that began to express GFP, around 1.5% and 1.0%, respectively (Figure 1B, C). HMGA1 overexpression also induced SOX2 expression (Figure 1C).

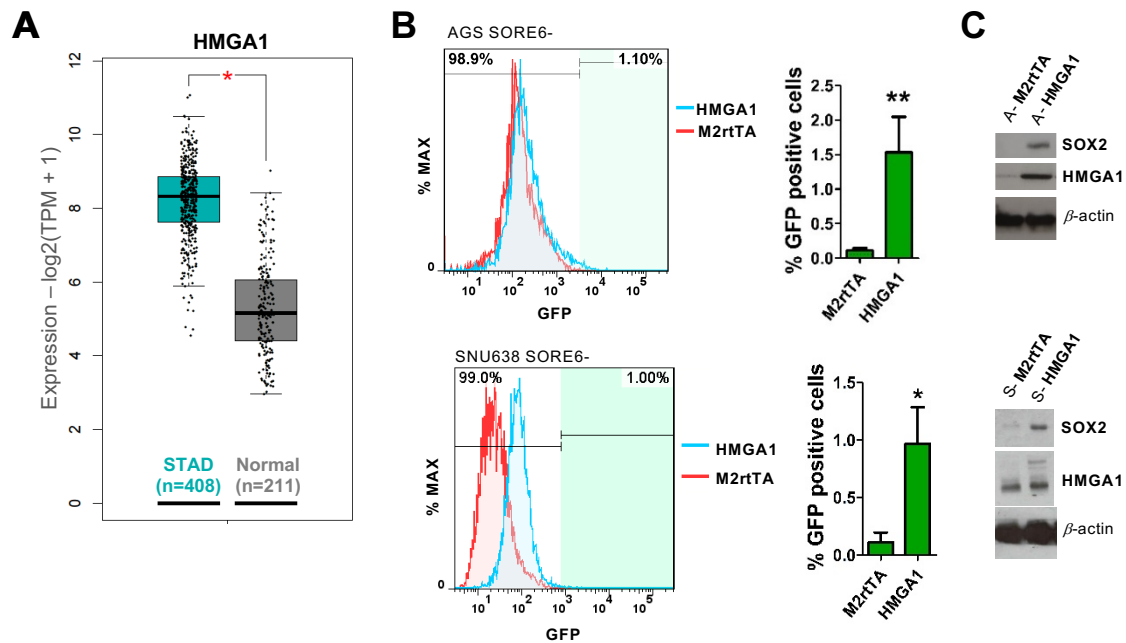


Figure 1 - HMGA1 overexpression efficiently activates the SORE6-GFP reporter system.

(A) Validation of the expression levels of HMGA1 in gastric cancer (STAD - stomach adenocarcinoma) (n = 408) and normal tissues (n = 211), based on TCGA and GTEx data in GEPIA2. (B) Percentage of GFP positive cells in AGS SORE6- and SNU638 SORE6- cells transduced with the DOX-inducible lentiviral vector pFUW-tetO-HMGA1 (HMGA1) and/or pFUW-M2rtTA (M2rtTA). (C) SOX2 and HMGA1 expression, evaluated by western blot, in AGS SORE6- and SNU638 SORE6- cells transduced with HMGA1. Cells transduced with M2rtTA were used as negative control for gating assessment. Results are mean ± SD of at least three independent experiments. Significant differences (* p ≤ 0.05; ** p ≤ 0.01).

3.2. HMGA1 GFP+ cells are enriched in stemness markers expression

We next explored the differences between cells that became GFP+ and those that remained GFP-, following overexpression of HMGA1 in SORE6- cells. The objective was to try to understand whether GFP+ cells presented cancer stem-like characteristics. Thus, we sorted the cells, by fluorescence-activated cell sorting (FACS), into two cell populations according to GFP expression (HMGA1 GFP- and HMGA1 GFP+) to compare the expression of stemness markers, and their characteristics based on functional assays (Figure 2A). To isolate a purer HMGA1 GFP+ cell population, we restricted the gate and collected cells from 0.442% and 0.684% GFP+ cells from AGS SORE6- and SNU638 SORE6- cells transduced with the HMGA1 vector, respectively (Figure 2B). Subsequently, when cells reached approximately 80% confluency, we analyzed the expression of relevant transcription factors. HMGA1 GFP+ cells, from both cell lines, showed significantly greater

expression of SOX2, C-MYC and KLF4 compared to HMGA1 GFP- cells (Figure 2C, D). Additionally, OCT4 was also upregulated in SNU638 SORE6- HMGA1 GFP+ cells compared to HMGA1 GFP- (Figure 2D). Although HMGA1 GFP+ cells showed a significant enrichment in the expression of several transcription factors compared to HMGA1 GFP-, HMGA1 expression was not significantly different between both cell populations (Figure 2D).

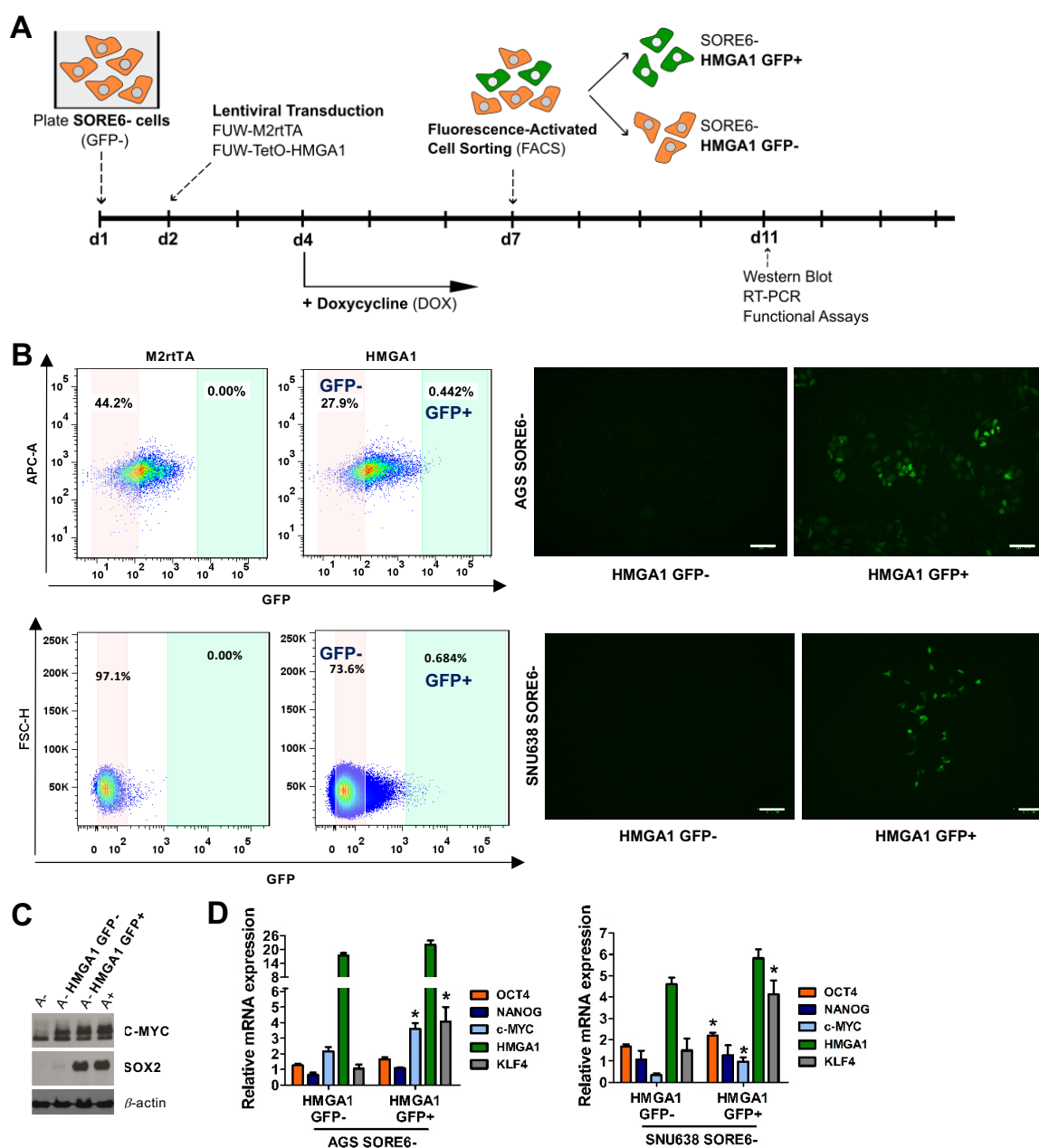


Figure 2 – HMGA1 GFP+ cells are enriched in stemness biomarkers. (A) Schematic representation of the protocol used to obtain HMGA1 GFP+ cells. AGS SORE6- and SNU638 SORE6- cells were plated and allowed to attach (day 1 - d1). Cells were transduced with HMGA1 and M2rtTA (d2), and doxycycline (DOX) was added (d4) to induce HMGA1 expression. Fluorescence-activated cell sorting (FACS) was performed based on GFP expression (d7) and

cells were characterized by western blot, RT-qPCR and functional assays (d11). **(B)** FACS of two AGS SORE6- and SNU638 SORE6- cell subpopulations, HMGA1 GFP+ cells (with GFP expression) and HMGA1 GFP- cells (without GFP expression), followed by fluorescence images of the respective populations sorted. Scale bar = 100 μ m. APC-A and FSC-H correspond to an empty channel used to exclude autofluorescent cells from the analysis. **(C)** SOX2 and C-MYC expression in AGS SORE6+, AGS SORE6-, AGS SORE6- HMGA GFP+ and HMGA GFP- cells, evaluated by Western Blot. **(D)** Real-time PCR analysis of mRNA levels of OCT4, HMGA1, C-MYC, NANOG and KLF4 in HMGA1 GFP+ and HMGA1 GFP- cells. Real-time PCR results were normalized to 18S expression. β -actin was used as an internal control. Cells transduced with M2rtTA were used as negative control for gating assessment. Results are mean $\pm$ SD of at least three independent experiments minus real-time PCR results from SNU638 SORE6- HMGA1 GFP+ and HMGA1 GFP- in which only two independent experiments were performed. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$).

3.3. HMGA1 GFP+ cells are more proliferative and resistant to 5-FU treatment

As stated before, CSCs have a high capacity for self-renewal, greater proliferative capacity and resistance to chemotherapeutic agents. When performing a colony formation assay, that is a cell survival assay based on the ability of a single cell to form a colony, we observed that HMGA1 GFP+ cells displayed increased ability to form colonies when compared with HMGA1 GFP- cells (Figure 3A). This difference between HMGA1 GFP- and HMGA1 GFP+ cells was also supported by the BrdU incorporation assay. HMGA1 GFP+ cells were significantly more proliferative than HMGA1 GFP- cells (Figure 3B). Additionally, cells were incubated with 5-FU and the percentage of apoptotic cells was determined by flow cytometry. HMGA1 GFP+ cells from both cell lines were significantly more resistant to 5-FU in comparison to the HMGA1 GFP- cells. After 48 h of treatment with 5-FU, around 50% of AGS SORE6- HMGA1 GFP+ cells and about 41% of SNU638 SORE6- HMGA1 GFP+ cells were in apoptosis (Figure 3C). Conversely, approximately 65% of AGS SORE6- HMGA1 GFP- cells and 55% of SNU638 SORE6- HMGA1 GFP- cells were apoptotic (Figure 3C). Thus, our results support that HMGA1 GFP+ cells, in addition to being enriched in stemness markers, also exhibited characteristics of CSCs.

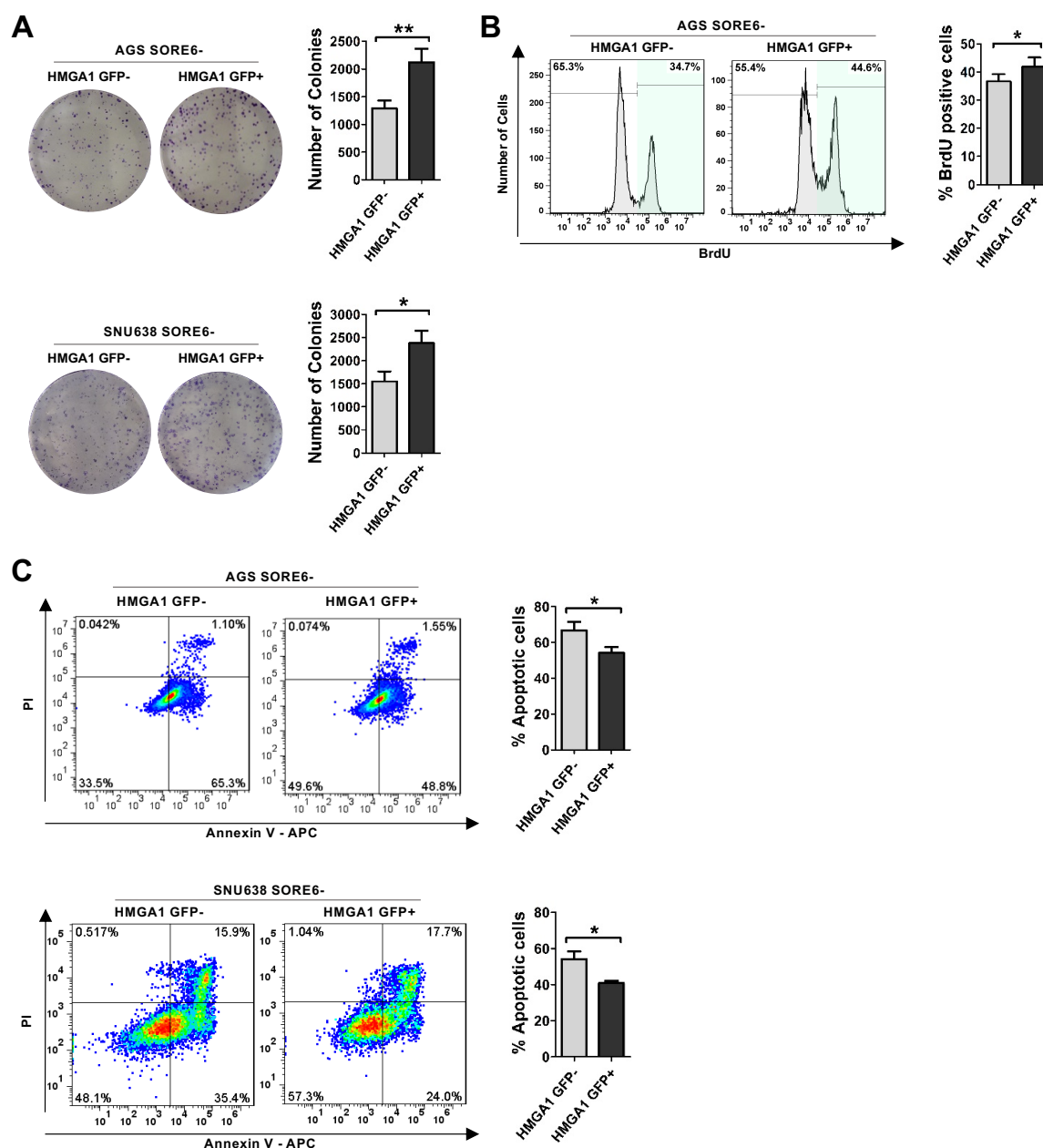


Figure 3 - HMGA1 GFP+ cells are more proliferative and resistant to 5-FU treatment. (A) Colony formation ability of HMGA1 GFP- and HMGA1 GFP+, representative images of the colonies are shown. **(B)** Flow cytometry analysis of BrdU incorporation in AGS SORE6- HMGA1 GFP- and HMGA1 GFP+ to evaluate the proliferation ability of each cell line. **(C)** Annexin V/propidium iodide (PI) assay results after flow cytometry analysis of HMGA1 GFP- and HMGA1 GFP+, from both cell lines, after 48 h treatment with 4.5 $\mu\text{g/mL}$ 5-FU. DMSO was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$).

3.4. HMGA1 promotes the biogenesis of cancer stem-like cells from non-CSCs

Recently, we uncovered that the antifungal agent CPX can generate gastric cancer stem-like cells by the upregulation of SOX2 expression in non-CSCs (29). Our previous results also revealed that CPX treatment led to increased HMGA1 expression (29). In order to further explore the role of HMGA1 in promoting the gastric cancer stem-like cell phenotype, we assessed whether it is relevant in the context of CPX treatment. For that, we treated AGS SORE6- cells with CPX and studied HMGA1 expression. Indeed, CPX treatment induces the expression of HMGA1 and this induction is dose-dependent (Figure 4A, B). Next, we treated HMGA1 GFP+ and HMGA1 GFP- cells with 8 μ M CPX for 48 h. Our results showed that when treated with 8 μ M CPX for 48 h, about 10% of HMGA1 GFP- started to express GFP and HMGA1 GFP+ cells increased GFP expression to about 15% (Figure 4C). These results show that the expression of HMGA1 facilitates the activation of the SORE6-GFP reporter system after exposure to CPX.

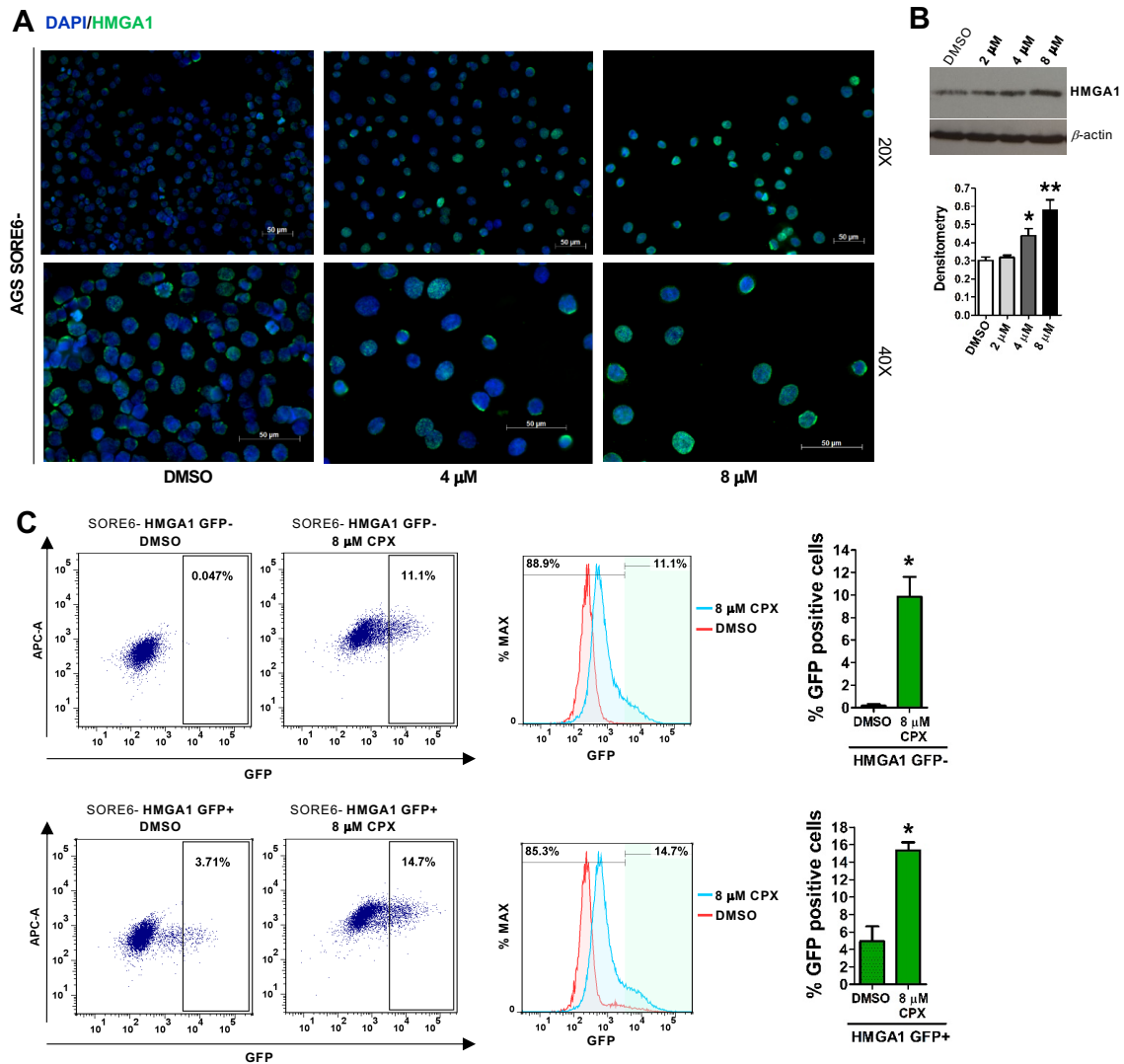


Figure 4 - Overexpression of HMGA1 improves CPX's ability to reprogram non-CSCs into CSCs. **(A)** Representative immunofluorescence of HMGA1 expression in AGS SORE6- cells after treatment with 4 or 8 μ M of CPX for 48h. Scale bar = 50 μ m. **(B)** HMGA1 expression in AGS SORE6- cells treated with 2, 4 or 8 μ M of CPX for 48 h, evaluated by western blot, followed by densitometry data. **(C)** Percentage of GFP positive cells in AGS SORE6- HMGA1 GFP- and HMGA1 GFP+ cells treated with 8 μ M of CPX for 48h. APC-A corresponds to an empty channel used to exclude autofluorescent cells from the analysis. β -actin was used as an internal control. DMSO was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$).

3.5. HMGA1 GFP+ cells are more sensitive to monensin

Previously, we showed that treatment with 8 μ M of monensin for 48 h selectively kills gastric cancer stem-like cells (28). In order to explore whether HMGA1 GFP+ cells are also more sensitive to monensin compared to HMGA1 GFP-, we treated the cells with 8 μ M monensin for 48 h. Our results showed that HMGA1 GFP+ cells, derived from AGS SORE6- and SNU638 SORE6- cells were significantly more sensitive to monensin treatment than HMGA1 GFP- cells. After treatment, about 48% of AGS SORE6- HMGA1 GFP- cells and about 54% of SNU638 SORE6- HMGA1 GFP- cells were viable (Figure 5A). Nevertheless, the percentage of cell viability in AGS SORE6- HMGA1 GFP+ cells and SNU638 SORE6- HMGA1 GFP+ cells were around 23% and 31%, respectively (Figure 5A). In the previous study, we also reported that the gastric cancer stem-like cells divide asymmetrically giving rise to GFP+ and GFP- cells, that is, they recapitulate the original lineage (28). Thus, after some time the population of GFP+ cells decreased. Here the same happened, after a few weeks, the HMGA1 GFP+ cell population became a heterogeneous cell population, having both GFP+ and GFP- cells (data not shown). Therefore, to be sure that GFP+ cells were directly affected by monensin treatment, we treated the cells and analyzed GFP expression by flow cytometry. After treatment with 8 μ M monensin for 48 h, we found that the percentage of GFP+ cells in AGS SORE6- HMGA1 GFP+ significantly decreased from around 8% to approximately 1% (Figure 5B). Curiously, monensin treatment appeared to induce GFP expression in AGS SORE6- HMGA1 GFP-, however, the values are not statistically significant (Figure 5B).

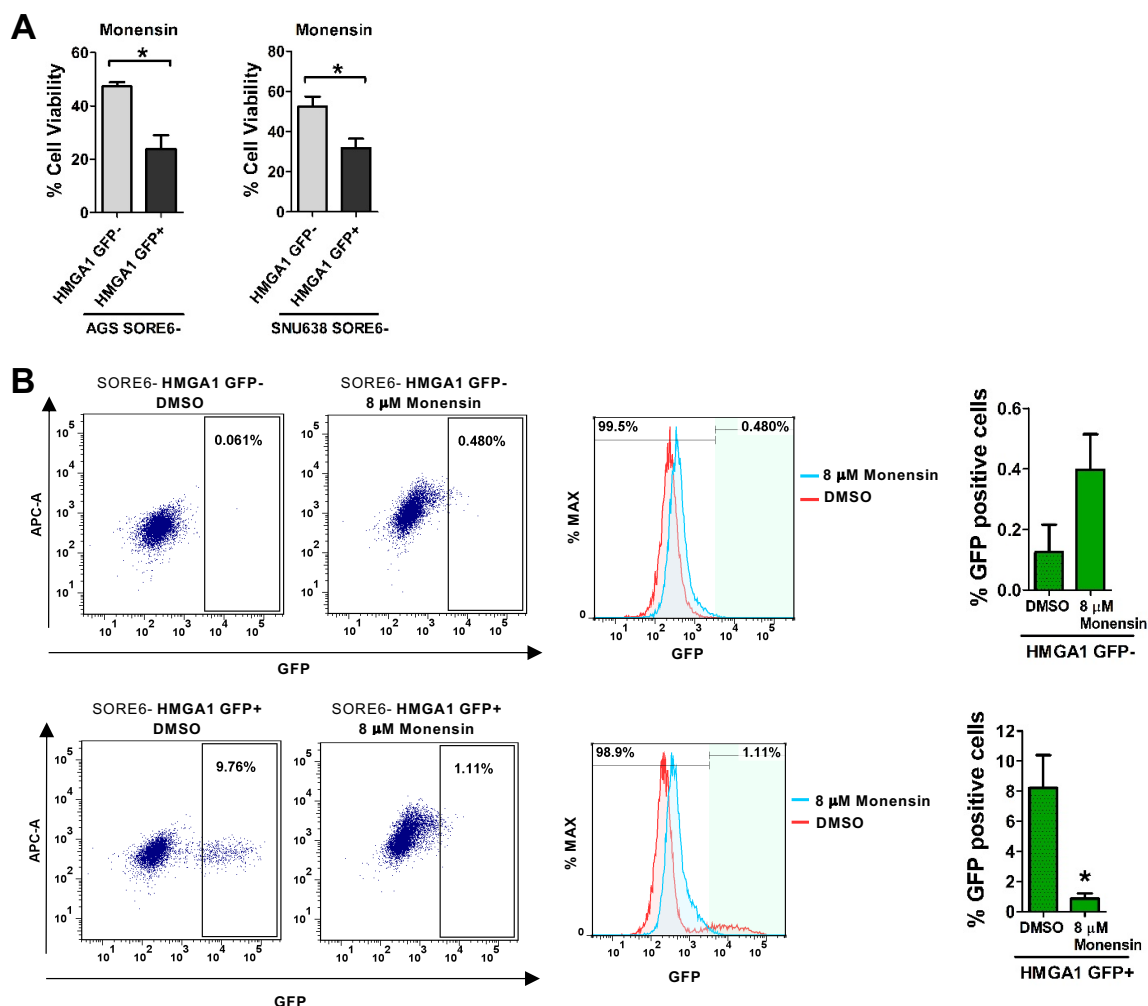


Figure 5 – HMGA1 GFP+ cells are sensitive to monensin, as are gastric cancer stem-like cells. (A) Cell viability of HMGA1 GFP- and HMGA1 GFP+, from AGS SORE6- and SNU638 SORE6- cells, after treatment with 8 μ M of monensin for 48 h. **(B)** Percentage of GFP positive cells in AGS SORE6- HMGA1 GFP- and HMGA1 GFP+ after treatment with 8 μ M of monensin for 48 h. APC-A corresponds to an empty channel used to exclude autofluorescent cells from the analysis. DMSO was used as a negative control. Results are mean $\pm$ SD of at least three independent experiments. Significant differences (* $p \leq 0.05$).

4. Discussion

Gastric cancer remains a serious health problem worldwide (1). The fact that CSCs are responsible for tumor progression, recurrence, and resistance to therapy has made them a crucial target in cancer treatment. However, the molecular circuitries behind their stem-like phenotype are not fully understood. Several transcription factors have been pointed out as potential markers of CSCs, such as SOX2, OCT4 and NANOG (33). Using the SORE6-GFP lentiviral reporter system, which is based on SOX2 and/or OCT4 expression, we isolated

gastric cancer stem-like cells (SORE6+) and non-CSCs (SORE6-) (27, 28). Our previous data revealed that the transcription factor SOX2 plays a key role in the stem-like properties of gastric CSCs (28). However, the upstream network of transcription regulators involved in SOX2 expression and CSCs reprogramming is not fully characterized. For example, the transcription factor HMGA1 sparked interest in stemness after promoting the conversion of non-stem cells into stem cells when added to the SOX2-OCT4-C-MYC-KLF4 transcription factor Yamanaka's cocktail (15). HMGA1 encodes a chromatin-associated protein that participate in DNA repair, transcriptional regulation, cell proliferation, migration, drug resistance and stemness (34, 35). Additionally, it prevents embryonic stem cell differentiation by maintaining high levels of OCT4, NANOG, SOX2 and MYC (15). HMGA1 has also been implicated in tumor progression (21, 36, 37).

Using the RNA sequencing data present in TCGA and GTEx, we showed that HMGA1 is significantly more expressed in gastric cancer tissues compared to normal gastric tissues. Concordantly, Yang et al. confirmed that HMGA1 is more expressed in human gastric cancer cell lines in comparison to an immortalized normal gastric epithelial cell line (GES-1) (21). HMGA1 has also been implicated in the regulation of cancer stem-like features in ovarian cancer cells and circulating gastrointestinal stromal tumor cells (28, 29). Additionally, we showed previously that the level of HMGA1 was significantly higher in SORE6+ cells compared to SORE6- cells (29). In order to find out whether HMGA1 expression impacts the phenotype of gastric CSCs, we overexpressed it in non-CSCs. As SORE6- cells lack GFP expression, we used the SORE6-GFP reporter system incorporated in these cells as a readout for the induction of a cancer stem-like state. The overexpression of HMGA1 was able to induce SOX2 expression in SORE6- cells, activating the SORE6-GFP reporter system in some cells. Since not all the cells became GFP+, we sorted the cells into HMGA1 GFP- and HMGA1 GFP+ cell populations to understand the difference between these cells. Our results showed that HMGA1 GFP+ cells were enriched in stemness-related transcriptional factors compared to HMGA1 GFP- cells. The involvement of HMGA1 in the expression of several transcription factors related with stemness has been demonstrated in several studies. Shah et al. reported that human embryonic stem cells transduced with the HMGA1 lentivirus displayed increased levels of SOX2 and C-MYC (15). Other studies have also demonstrated that HMGA1 regulates and promotes SOX2 expression through binding to its promoter (38, 39). HMGA1 has also been reported to induce C-MYC expression in gastric cancer (23). However, C-MYC can also induce HMGA1 expression, creating a positive feedback loop between HMGA1 and C-MYC in gastric cancer cells (22). HMGA1 also appears to directly regulate KLF4 expression. Overexpression of HMGA1 in ovarian cancer cells increased several stemness-related

genes, including KLF4 (25). Induction of OCT4 expression has also been linked to HMGA1 expression (15, 39).

The present study shows that HMGA1 GFP⁺ cells display CSCs characteristics, namely greater ability to form colonies, higher proliferation activity and a superior resistance to 5-FU when compared with HMGA1 GFP⁻ cells. Accordingly, HMGA1 was demonstrated to promote gastric cancer growth and spread through metastasis (21, 40). In other tumor types, for instance in cervical and renal cancer cells, the overexpression of HMGA1 increased the ability to form colonies and improved proliferation, migration and invasion capability of the cells (37, 41), while the inhibition of HMGA1 expression blocked cell proliferation and the colony formation (36, 42, 43). In thyroid and colon cancer cells, HMGA1 overexpression confers resistance to 5-FU (44). Wang reported that transfection of 5-FU-resistant AGS cells with miR-195 led to inhibition of HMGA1 expression, increasing the cells' sensitivity to 5-FU (45). Thus, in gastric cancer, HMGA1 expression appears to be associated with 5-FU resistance. Due to the resistance to therapy demonstrated by CSCs, several studies have sought to find or develop new methods and therapies capable of selectively killing these cells. Salinomycin and monensin, both polyether ionophore antibiotics were demonstrated to target gastric CSCs (28, 46). In accordance, we observed in this study that HMGA1 GFP⁺ cells are more sensitive to monensin treatment compared to HMGA1 GFP⁻ cells. In particular, GFP⁺ cells within the heterogeneous population of HMGA1 GFP⁺ cells were most affected by the treatment, revealing that HMGA1 GFP⁺ cells may share the same molecules involved in drug resistance/sensitivity as SORE6⁺ cells.

We previously disclosed that CPX can promote the biogenesis of gastric cancer stem-like cells and that these cells are enriched in HMGA1 (29). Here, we confirmed that CPX treatment induced HMGA1 expression in a dose-dependent manner. Interestingly, the cellular reprogramming of somatic cells to iPS cells with the Yamanaka factors is improved by the expression of HMGA1 (15). Our results appear to support this report. The presence of HMGA1 expression in the cells treated with CPX increased its ability to induce GFP⁺ cells, this is, to generate gastric cancer stem-like cells.

5. Conclusion

Our results indicated that HMGA1 belongs to the network of transcription factors responsible for the characteristics of gastric cancer stem-like cells. The overexpression of HMGA1 in non-CSCs activated the SORE6-GFP reporter system through upregulation of SOX2 expression. We showed that HMGA1 GFP⁺ cells were enriched in SOX2, C-MYC and KLF4. In accordance, HMGA1 GFP⁺ cells displayed greater ability to proliferate and

form colonies, and increased resistance to 5-FU compared to HMGA1 GFP⁻ cells. Furthermore, we demonstrated that HMGA1 expression potentiates CPX-mediated cellular reprogramming of gastric non-CSCs into cancer stem-like cells. And we also found that HMGA1 GFP⁺ cells are more sensitive to monensin than HMGA1 GFP⁻ cells. Although additional studies are needed, our findings suggest that HMGA1 plays an essential role in the phenotype and characteristics of gastric CSCs and also suggest that HMGA1 participates in cellular reprogramming toward a cancer stem-like state. Thus, targeting HMGA1 expression could be a promising approach in the treatment of gastric cancer.

Author Contributions

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

Funding

This research was funded by the project Norte-01-0145-FEDER-000051-“Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets”, supported by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, via the European Regional Development Fund (FEDER). Diana Pádua acknowledges Fundação para a Ciência e a Tecnologia (FCT) for financial support via a Ph.D. fellowship (SFRH/BD/146186/2019), co-sponsored by Fundo Social Europeu (FSE) via the North Portugal Regional Operational Programme. Patrícia Mesquita acknowledges financial support via Programa Operacional Regional do Norte and European Regional Development Fund under the project “The Porto Comprehensive Cancer Center” with the reference NORTE-01-0145-FEDER-072678 - Consórcio PORTO.CCC—Porto.Comprehensive Cancer Center.

Data Availability Statement

The data obtained and analyzed during this work are presented in the published article together with its Supplementary Materials. The data are also available from the authors upon request.

Acknowledgments

The authors acknowledge the support of the i3S Scientific Platform BioSciences Screening Advanced Light Microscopy, member of the national infrastructures PT-OPENSCREEN (NORTE-01-0145-FEDER-085468) and PPBI—Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122). The authors also acknowledge the support of the Cell Culture and Genotyping, Genomics, and Biochemical and Biophysical Technologies i3S Scientific Platforms. We thank Mafalda Sousa for having developed the custom MATLAB script.

Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER V

HMGA1 HAS PREDICTIVE VALUE IN RESPONSE TO CHEMOTHERAPY IN GASTRIC CANCER

This chapter contains the following publication:

Pádua, D., Pinto, D. F., Figueira, P., Pereira, C. F., Almeida, R., & Mesquita, P. (2021). *HMGA1 Has Predictive Value in Response to Chemotherapy in Gastric Cancer*. *Current Oncology*, 29(1), 56–67.

<https://doi.org/10.3390/curroncol29010005>

In Chapter IV, we verified that the overexpression of HMGA1 can induce the expression of SOX2 and consequently activate the SORE6-GFP reporter system in SORE6- cells. Thus, our results revealed that HMGA1 has a potential role in inducing the phenotype of gastric CSCs. Taking these results into account, and since little is known about the clinical impact of HMGA1 expression in this context, we decided to evaluate the clinical value of HMGA1 in gastric cancer. To this end, we used a single hospital series (n = 323) of gastric adenocarcinoma cases (stages I to IV) treated surgically at Centro Hospitalar São João (CHSJ), Porto, Portugal. For all patients, tumor tissue data, clinicopathological and treatment data, as well as follow-up information were available.

In this Chapter, *HMGA1 Has Predictive Value in Response to Chemotherapy in Gastric Cancer*, we report the results obtained after immunohistochemistry analysis of the nuclear expression of HMGA1 in a series of 323 samples of gastric cancer, the largest series of tumors used to date for this purpose and the most complete in terms of clinicopathological characteristics, survival data, and treatment data. Although we did not find a significant correlation between HMGA1 and SOX2 expression, we disclosed that HMGA1 expression is a useful marker for predicting response to chemotherapy in gastric cancer. Our results unequivocally showed that patients with high levels of HMGA1 expression benefit more from chemotherapy compared to those with low levels of this protein.

Article

HMGA1 Has Predictive Value in Response to Chemotherapy in Gastric Cancer

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Abstract: Gastric cancer is a serious health problem worldwide. Although its incidence is decreasing, the five-year survival rate remains low. Thus, it is essential to identify new biomarkers that could promote better diagnosis and treatment of patients with gastric cancer. High-mobility group AT-hook 1 (HMGA1) is a non-histone, chromatin-binding protein that has been found overexpressed in several tumor types. It has been correlated with invasion, metastasis, and drug resistance, leading to worse patient survival. The aim of this work was to evaluate the clinical value of HMGA1 in gastric cancer. HMGA1 expression was analyzed by immunohistochemistry in a single hospital series ($n = 323$) of gastric adenocarcinoma cases (stages I to IV) with clinicopathological and treatment data. In this series, HMGA1 expression showed no significant relevance as a prognostic biomarker. Nevertheless, a significantly better overall survival was observed in cases with high levels of HMGA1 when they were treated with chemotherapy, compared to the nontreated ones, implying that they can benefit more from treatment than patients with low expression of HMGA1. We thereby show for the first time that HMGA1 expression has a substantial value as a biomarker of response to chemotherapy in gastric cancer.

Keywords: HMGA1; gastric cancer; prognosis; predictive value; chemotherapy



Citation: Pádua, D.; Pinto, D.F.; Figueira, P.; Pereira, C.F.; Almeida, R.; Mesquita, P. HMGA1 Has Predictive Value in Response to Chemotherapy in Gastric Cancer. *Curr. Oncol.* **2022**, *29*, 56–67. <https://doi.org/10.3390/curroncol29010005>

Received: 17 November 2021

Accepted: 21 December 2021

Published: 23 December 2021

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1. Introduction

Gastric cancer (GC) remains one of the most commonly diagnosed cancers and the fourth leading cause of global cancer mortality [1]. The majority of GCs are adenocarcinoma (90% to 95%). According to the Laurén classification system, the gastric adenocarcinomas can be histologically classified as intestinal, diffuse and mixed type, depending on tissue architecture and glandular patterns. The diffuse-type involves non-cohesive and poorly differentiated tumors, with no gland formation, while intestinal-type tumors are moderate to differentiated tumors, having glandular structures. The mixed type presents features from both the diffuse and intestinal types [2,3]. GC treatment options depend on the stage of the disease at the time of diagnosis. At the early stages, surgical resection of the tumor is the preferred treatment [4]. For stages IB–III, surgery is recommended along with perioperative or adjuvant chemotherapy in order to improve overall patient survival due to the probable relapse after resection [4]. Yet, a significant number of patients have advanced disease at diagnosis, and, consequently, surgery is no longer an option [5]. Therefore,

for stage IV, first-line chemotherapy with doublet or triplet platinum/fluoropyrimidine combinations or second-line chemotherapy using taxane, ramucirumab or irinotecan alone or in combination with paclitaxel are recommended [4]. Despite all the improvements registered in the diagnosis and treatment of GC in the last decades, patient prognosis remains poor [5,6]. In Europe, five-year survival rates are enormously diverse between different countries, with a maximum value of 37.5% obtained when best practices are used [7]. The heterogeneity of GC at the molecular level plays an important role in terms of tumor aggressiveness, regardless of tumor extension or tumor type [8,9]. This reveals an urgent need to better understand the disease in order to improve diagnostic techniques and treatments. The validation of new biomarkers can allow for a more personalized practice by making it possible to distinguish between patients who may benefit more from systemic treatment at a specific stage of the disease [10,11]. These biomarkers should comprise transcription factors (TFs), as they are essential in the control of regulatory gene networks and cell fate, and their normal activity is usually altered in different types of cancer [11–13].

The transcription factor high-mobility group AT-hook 1 (HMGA1) is a non-histone, chromatin-binding protein with an important role in chromatin remodeling [14]. HMGA1 binds to nuclear chromatin at AT-rich regions located in the minor grooves of DNA, where it bends the chromatin, allowing additional TFs to bind, thereby influencing gene regulation [14,15]. It is involved in DNA repair, cell cycle progression, cell proliferation, cell metabolism, and apoptosis [16–18]. Additionally, HMGA1 prevents embryonic stem cell differentiation by maintaining high levels of OCT4, NANOG, SOX2, and MYC [19]. Consequently, HMGA1 suppression decreased the expression of the pluripotency genes [20]. The HMGA1 protein is also found overexpressed in numerous aggressive human cancers [21–24]. It acts as an oncogene, and its high expression in cancer has been implicated in malignant transformation, tumor progression, and metastasis, while correlated with unfavorable clinical outcomes of the patients [23,25–30]. Accordingly, studies using tumor tissue specimens revealed that high HMGA1 expression is associated with a poor differentiation state and advanced disease or high-grade cancers [31–33]. Although the specific role and molecular mechanisms of HMGA1 in cancer have not been fully uncovered, studies have revealed that HMGA1 overexpression induced cancer cell proliferation, migration/invasion, and contributed to drug resistance in several tumors, including GC [16–18,25,34–41]. In GC, HMGA1 was also shown to stimulate epithelial–mesenchymal transition (EMT), thereby favoring a malignant progression [27]. It was also shown to participate in the regulation of aerobic glycolysis by regulating C-MYC expression [17]. However, the relevance of HMGA1 as a predictive biomarker with potential clinical applications has not been assessed in GC. Thus, the aim of this study was to investigate the clinical value of HMGA1 expression in a robust series of gastric adenocarcinoma cases with clinicopathological and treatment data. The importance of HMGA1 in predicting prognosis and response to therapy in GC patients was assessed by analyzing its expression in 323 cases from a consecutive, single hospital series. We observed that patients having tumors with high expression of HMGA1 benefit significantly more from chemotherapy compared to those with low expression. This observation can irrefutably contribute to a more efficient disease monitoring and treatment planning in GC patients.

2. Materials and Methods

2.1. Patients

Histological materials from 323 GC patients, mean age 67.68 ± 11.85 , were collected from surgical specimens of consecutive cases of gastric adenocarcinoma surgically treated between January 2008 and December 2014 at Centro Hospitalar São João (CHSJ), Porto, Portugal. For all patients ($n = 323$), tumor tissue, clinicopathological and treatment data, as well as follow-up information were available. In addition to surgery, 121 patients out of 323 received platinum- and/or fluoropyrimidine-based chemotherapy, mostly as adjuvant treatments. Relevant clinical information on this series is provided in Mesquita et al. [42] and in the Table S1.

2.2. Tissue Microarrays and Immunohistochemistry

Cores from formalin-fixed, paraffin-embedded tumor tissue blocks from the surgical specimens were used to construct tissue microarrays (TMAs). After construction of the TMAs, they were sectioned with a microtome (Microm HM 335 E) at a thickness of 4 μm , and the expression of HMGA1 was evaluated by immunohistochemistry (IHC) staining using standard protocols described in Mesquita et al. [42]. Rabbit recombinant monoclonal HMGA1 primary antibody (anti-HMGA1, 1:5000 dilution, Abcam ab252930, Cambridge, UK) was incubated overnight at 4 °C in a humidified chamber. Samples were considered HMGA1-low when they were negative for HMGA1 or it was present in less than 20% of the malignant cells and considered HMGA1-high otherwise, in agreement between two observers.

2.3. Statistical Analysis

We aimed to evaluate the association between the expression status of HMGA1 and the clinicopathological features of the tumors, for which we applied different statistical tests. To compare using the patient's age, we used Student's *t*-test. For gender, tumor growth pattern, vascular invasion, perineural invasion, and treatment, we applied Fisher's exact test (2-sides). For the Laurén classification and tumor node metastasis (TNM) staging, we used the chi-square test (χ^2). We also aimed to investigate the association between HMGA1 expression status and risk of death or relapse. This was done using the Kaplan-Meier method in order to generate plots and survival curves for five-year overall survival (OS—time from operation to death from any cause) and disease-free survival (DFS—time from surgery to the first event of either locoregional recurrence or metastasis or death from the same cancer) that were compared using the log-rank test. To assess whether HMGA1 expression could predict response to chemotherapy, OS and DFS plots were generated according to the expression status of this protein and administration or not of chemotherapy. The Cox proportional hazard model was used to calculate univariate and multivariate hazard ratios and confidence intervals for death. The clinicopathological parameters included in the multivariate models were selected based on their individual clinical relevance. Patients with incomplete data were excluded from the analyses. Statistical analysis of the data was performed using the IBM SPSS Statistics version 24 (IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant when *p* value < 0.05.

3. Results

3.1. Association of HMGA1 Expression with Clinicopathological Features in Gastric Carcinomas

HMGA1 expression was analyzed by IHC in 323 gastric carcinomas (Figure 1). Expression of HMGA1 was nuclear. High HMGA1 expression was observed in 223 (69%) gastric carcinoma cases (Figure 1A). HMGA1 was absent or low in 100 (31%) cases (Figure 1B). Clinicopathological features of the 323 GC cases and their association with HMGA1 expression are summarized in Table 1.

High levels of HMGA1 were significantly associated with tumors classified as intestinal (in 78% of the cases) or mixed (in 71% of the cases) according to the Laurén classification ($p < 0.001$), in opposition to tumors of the diffuse type (only in 44% of the cases) (Table 1). The other clinicopathological parameters, which included age at diagnosis, gender, growth pattern, TNM, and vascular and perineural invasion were not significantly associated with HMGA1 expression.

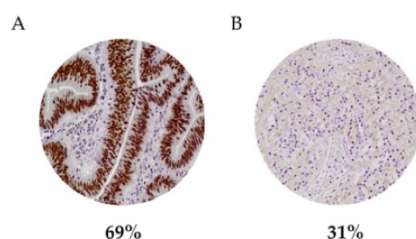


Figure 1. Representative immunostaining patterns for HMGA1 protein expression in two different GC cases and the respective percentages in the 323 gastric carcinomas evaluated (20× magnification). (A) GC case with high expression of HMGA1 in the nucleus (brown); 69% of gastric carcinomas express high levels of HMGA1 in the nucleus. (B) GC case with low HMGA1 expression; absence or low levels of HMGA1 were observed in 31% of gastric carcinoma cases. Tissues were counterstained with hematoxylin (purple).

Table 1. Clinicopathological association with HMGA1 expression in 323 patients diagnosed with GC.

	All Cases		HMGA1 High		HMGA1 Low		<i>p</i>
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
Patients	323		223	69.0	100	31.0	
Age							
Mean ± SD	67.68 ± 11.85		68.26 ± 11.61		66.39 ± 12.32		0.19
Range	32–95		33–95		32–87		
Gender							
Female	140	43.3	100	71.4	40	28.6	0.47
Male	183	56.7	123	67.2	60	32.8	
Laurén Classification							
Intestinal	151	46.8	117	77.5	34	22.5	<0.001 *
Diffuse	41	12.7	18	43.9	23	56.1	
Mixed	85	26.3	60	70.6	25	29.4	
Unclassified	46	14.2					
Growth Pattern							
Expansive	58	18.0	38	65.5	20	34.5	0.53
Infiltrative	252	78.0	176	69.8	76	30.2	
Unclassified	13	4.0					
TNM							
I	106	32.8	82	77.4	24	22.6	0.14
II	86	26.6	54	62.8	32	37.2	
III	68	21.1	46	67.6	22	32.4	
IV	63	19.5	41	65.1	22	34.9	
Vascular Invasion							
No	134	41.5	93	69.4	41	30.6	1.00
Yes	186	57.6	128	68.8	58	31.2	
ND	3	0.9					
Perineural Invasion							
No	167	51.7	120	71.9	47	28.1	0.28
Yes	156	48.3	103	66.0	53	34.0	
Chemotherapy treatment							
No	200	61.9	141	70.5	59	29.5	0.46
Yes	121	37.5	80	66.1	41	33.9	
ND	2	0.6					

Notes: *p* values (statistical significance threshold < 0.05) were obtained using Student's *t* test for the continuous variable, Fisher's exact test (2-sided) and Chi-square (χ^2) test for categorical variables; * comparisons with *p* < 0.05. ND = not determined; SD = standard deviation.

3.2. Evaluation of the Prognostic Significance of HMGA1 Expression in GC

To assess the relevance of HMGA1 as a biomarker in our series of 323 patients diagnosed with GC, we started by evaluating if HMGA1 expression predicted patient OS or DFS using the Kaplan-Meier method. HMGA1 showed no significant relevance as a prognostic biomarker (Figure 2A,B) when considering all the patients or when patients were stratified by the TNM stage (Figure 3A–D).

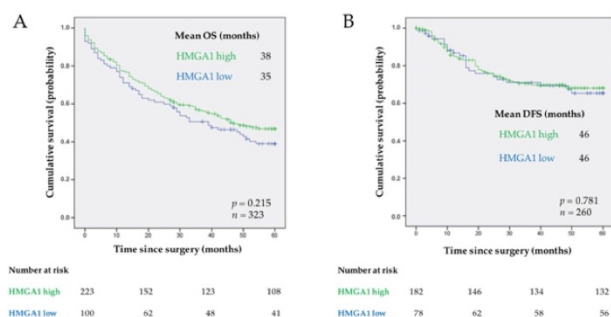


Figure 2. Kaplan-Meier curves showing the probability of (A) OS and (B) DFS in our series of patients with GC according to HMGA1 expression. Mean OS is shown for each subgroup of patients. The log-rank test was used to test for differences in survival between cases with high and low expression of HMGA1. *p* value is indicated. The number of patients at risk is specified for 0, 20, 40, and 60 months.

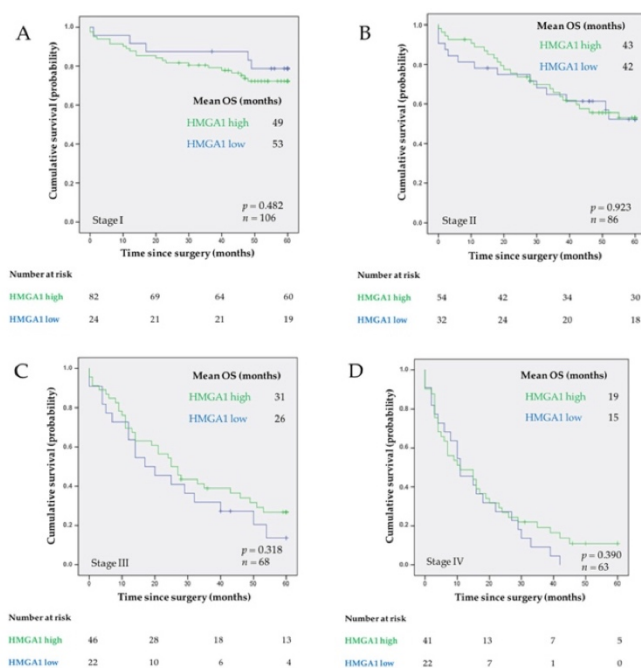


Figure 3. Kaplan-Meier curves showing the probability of OS according to HMGA1 expression in different stages of GC: (A) stage I, (B) stage II, (C) stage III, and (D) stage IV. Mean OS is shown for each subgroup of patients. The log-rank test was used to test for differences in survival between cases with high and low expression of HMGA1. *p* value is indicated. The number of patients at risk is specified for 0, 20, 40, and 60 months.

3.3. Evaluation of the Predictive Value of HMGA1 for Response to Chemotherapy in GC

Regarding treatment, 121 (37.5%) patients received chemotherapy, which improved OS [42]. The value of HMGA1 as a biomarker of response to chemotherapy was investigated. We observed a significantly better outcome in cases that had high levels of HMGA1 when they were treated with chemotherapy, comparing with those not treated (Figure 4A). The mean OS of patients with HMGA-high tumors treated with chemotherapy was 39 ± 2 months versus 24 ± 3 months in nontreated patients ($p < 0.001$), and indeed the combination of HMGA1-high expression and no treatment defined the group of patients with the worst outcome regarding OS. We did not observe a significant difference in cases with low levels of HMGA1, with or without chemotherapy (Figure 4B). Accordingly, in the universe of cases treated with chemotherapy, we saw a better outcome for the cases with high expression of HMGA1 (mean OS of 39 ± 2 months) compared to those with low expression (mean OS of 30 ± 3 months), although not significant (Figure 4C; $p = 0.051$). In the absence of chemotherapy, we did not observe a significant difference in OS between cases with different levels of HMGA1 expression (Figure 4D). The correlation between HMGA1 expression and adjuvant chemotherapy regarding DFS was also investigated (Figure S1), but we have not observed statistically significant differences.

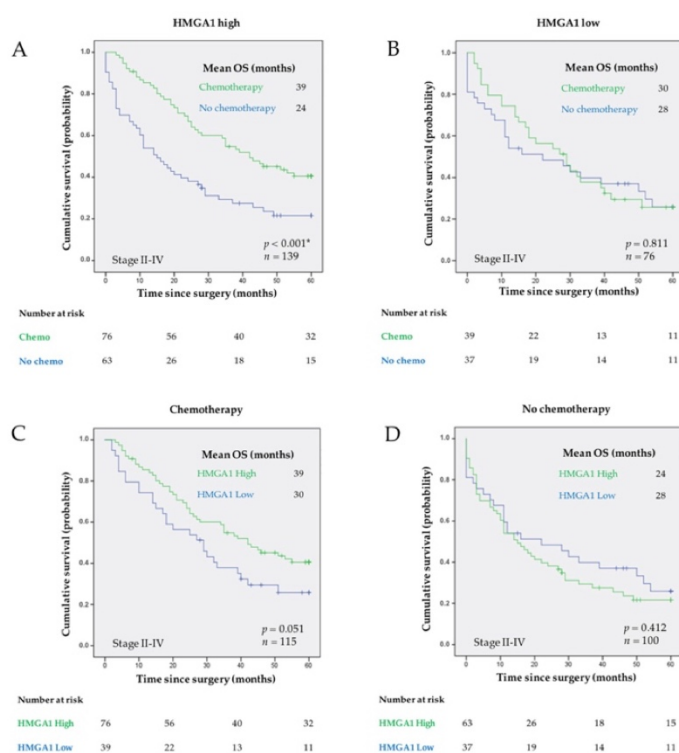


Figure 4. Kaplan-Meier curves showing the probability of OS according to treatment options in patients having tumors with: (A) high and (B) low expression of HMGA1, respectively. Alternatively, Kaplan-Meier curves showing the probability of OS according to levels of expression of HMGA1 in patients (C) treated or (D) not treated with chemotherapy. Mean OS is shown for each subgroup of patients. The log-rank test was used to test for differences in OS between treatment and absence of treatment (A,B) or between high and low levels of HMGA1 (C,D). p value is indicated. * comparisons with $p < 0.05$. The number of patients at risk is specified for 0, 20, 40, and 60 months.

Independently analyzing the cases diagnosed in each TNM stage—II to IV—we observe a clear and significant benefit in treating the tumors diagnosed in stages III and IV that have high expression of HMGA1 ($p < 0.001$ for both stages), whereas the same was not true for those presenting low expression (Figure 5A–F). In stage III, the mean OS for patients having tumors with high levels of HMGA1 was 40 ± 4 months in patients treated with adjuvant chemotherapy in addition to surgery, compared to 15 ± 4 months in patients not treated. In stage IV, patients having tumors with high levels of HMGA1 that were treated with palliative chemotherapy had a mean OS of 26 ± 4 months while those not treated had a mean OS of 6 ± 2 months.

Furthermore, HMGA1 expression was independently associated with a lower risk of death in a Cox multivariate analysis restricted to patients diagnosed in stages II–IV treated with chemotherapy and that includes relevant clinicopathological factors such as Laurén classification, growth pattern, TNM staging, and vascular and perineural invasion as covariates (Table 2; HR = 0.53; 95% CI 0.30–0.95; $p = 0.03$).

Table 2. Overall survival univariate and multivariate Cox regression analysis in gastric cancer patients diagnosed in stages II–IV and treated with chemotherapy.

	Number of Events	Univariate Analysis			Multivariate Analysis		
		HR	95% CI	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value
HMGA1							
Low	28	1			1		
High	44	0.63	0.39–1.01	0.06	0.53	0.30–0.95	0.03 *
Laurén Classification							
Intestinal	23	1			1		
Diffuse	15	1.75	0.91–3.36	0.09	1.33	0.61–2.89	0.47
Mixed	23	1.51	0.84–2.27	0.17	1.06	0.56–1.98	0.87
Growth pattern							
Infiltrative	65	1			1		
Expansive	5	0.74	0.30–1.83	0.51	0.82	0.31–2.16	0.68
TNM							
II	14	1			1		
III	27	2.04	1.07–3.89	0.03 *	2.03	0.96–4.29	0.06
IV	32	4.40	2.31–8.38	<0.001 *	4.41	2.08–9.34	<0.01 *
Vascular Invasion							
No	20	1			1		
Yes	52	1.34	0.80–2.24	0.27	1.81	0.97–3.38	0.06
Perineural Invasion							
No	20	1			1		
Yes	52	1.65	0.99–2.78	0.06	1.09	0.58–2.03	0.80

Notes: *p* values (statistical significance threshold < 0.05) were obtained using univariate and multivariate Cox proportional hazards regression analysis (Wald); * comparisons with $p < 0.05$. HR = Hazard-ratio; CI = Confidence interval.

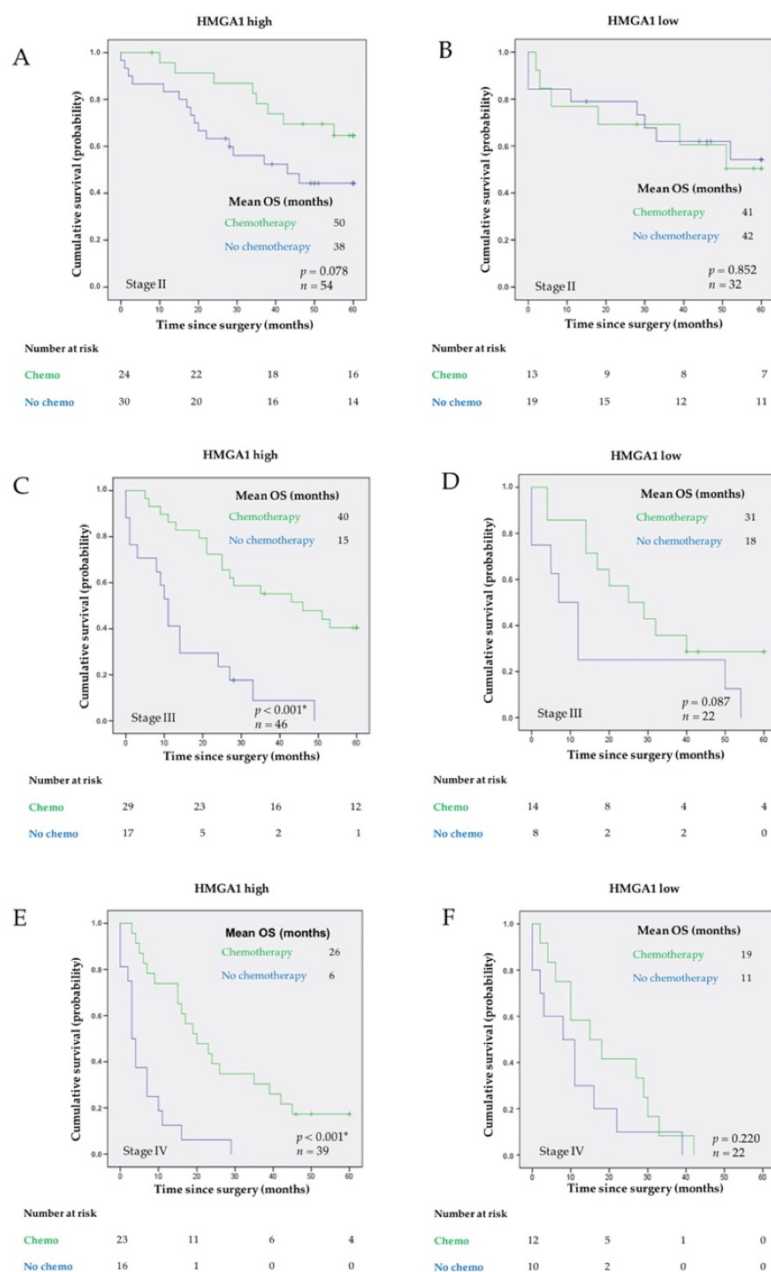


Figure 5. Kaplan-Meier curves showing the probability of OS according to treatment options in patients having tumors with: (A,C,E) high and (B,D,F) low expression of HMGA1, in patients diagnosed in: (A,B) stage II, (C,D) stage III, and (E,F) stage IV, respectively. Mean OS is shown for each subgroup of patients. The log-rank test was used to test for differences in survival between treatment and absence of treatment with chemotherapy. p value is indicated. * comparisons with $p < 0.05$. The number of patients at risk is specified for 0, 20, 40, and 60 months.

4. Discussion

In the present study, we determined the value of nuclear HMGA1 expression in predicting GC's prognosis and response to treatment, in addition to its association with clinicopathological features of the tumors. This assessment was performed using a series of 323 gastric carcinomas, the largest series of tumors used to date for this purpose and the most complete in terms of clinicopathological features, survival data, and treatment data.

We found high levels of nuclear expression of HMGA1 in 69% of the tumors, which is comparable to those observed in all the reports published so far in the series of GC cases: 59% [28], 75% [43], and 74% [30]. An association between high expression of HMGA1 and Laurén intestinal and mixed-type tumors was observed in our series, and this agrees with previous reports of HMGA1 expression in gastric glands [18]. None of the other clinicopathological features were associated with HMGA1 expression. Accordingly, Nam et al. (2003) and Jun et al. (2015) reported no association at all between HMGA1 expression and clinicopathological features evaluated in a series of 62 and 110 GC cases, respectively, despite their analysis not including Laurén mixed-type tumors [30,43]. Contrarily, Yang et al. (2021) observed that increased HMGA1 expression was closely related with differentiation, lymph node metastasis, tumor size, and TNM stage, but this study was performed in a small series with only 51 GC samples [28].

To the best of our knowledge, our study is the first to identify HMGA1 expression as a useful marker to predict response to chemotherapy in GC. Our results unequivocally showed that patients with high levels of HMGA1 expression in the tumor had remarkably better OS rates when undergoing treatment with chemotherapy. This suggests that these patients can benefit more from this type of treatment than patients with low HMGA1 expression, where no significant difference was observed between treated and untreated patients. In the literature, there are several reports in cancer cell lines describing the role of HMGA1 expression in conferring resistance to antineoplastic drugs [44–46], including in GC [41]. These observations are in apparent contradiction with our results, if we consider only the association of HMGA1 with chemoresistance [47]. However, when we consider that HMGA1-high expression has been associated with features of higher malignancy in GC, such as increased proliferation, migration, metastasis, and EMT [18,27,28], the observation that HMGA1-tumors have better outcomes when subjected to more aggressive treatments is not surprising. Our results suggest that this parameter should be considered to determine which patients may benefit more from intensive chemotherapy management in an attempt to perform a more personalized medicine. This conclusion has a great impact because it advises for the treatment with chemotherapy of the 65% of GC patients with high HMGA1 expression diagnosed in stages II–IV. Considering only the cases with high expression of HMGA1 detected in stages III and IV, where the significant and most striking differences were obtained in mean OS values, our data supports the selection of 40% of the GC patients diagnosed in all tumor stages, except stage I, for treatment. Besides this role we describe for HMGA1 in predicting chemotherapy response, the role described for HMGA1 as a glycolysis regulator in gastric cancer suggests that the HMGA1-c-Myc-glycolysis axis may also be used as a new target in cancer therapy in combination with commonly used chemotherapeutic agents [17].

High expression of HMGA1 has already been correlated with worse survival in GC patients but only in studies that used series with a relatively small number of cases [28,43] or TCGA/GEPIA datasets [17,27]. High expression of another member of the family, HMGA2, has also been reported as predicting bad prognoses in two series of Asian GC patients [43,48]. However, in our series, we did not observe a prognostic value for HMGA1. This somewhat intriguing result can only be explained by our observation that chemotherapy modifies the course of the disease for patients having high expression of HMGA1 in the tumor, increasing the OS of these cases. In fact, when we considered only the patients treated with chemotherapy, we observed that HMGA1 expression lowers the risk of death to one half, independently from relevant clinicopathological factors.

5. Conclusions

Additional studies are needed, but our observation that GC patients with high levels of HMGA1 expression in the tumor benefit more from being treated with chemotherapy, should advise the selection of these patients for treatment, with expected improvements of their overall survival.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/curroncol29010005/s1>, Figure S1: Kaplan-Meier curves showing the probability of DFS according to treatment options, Table S1: Chemotherapy regimens used in the 121 treated GC patients, namely in cases having either high or low expression of HMGA1.

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

Funding: This research was funded by FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020 and by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274); Project POCI-01-0145-FEDER-029017 funded by FCT and project Norte-01-0145-FEDER-000051—“Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets”, supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER). Diana Pádua acknowledges FCT for financial support through a PhD fellowship (SFRH/BD/146186/2019), co-sponsored by FSE (Fundo Social Europeu) through NORTE2020.

Institutional Review Board Statement: The study was approved by the ethics committee of CHSJ (Ethics Committee references CES 122/15 and CES 117/18).

Informed Consent Statement: Informed consent was obtained from all patients.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Acknowledgments: The authors wish to acknowledge the tumor and tissue bank at Centro Hospitalar de São João (CHSJ), Porto, Portugal for providing all the means to collect the human tissue samples included in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary Material

HMGA1 has Predictive Value in Response to Chemotherapy in Gastric Cancer

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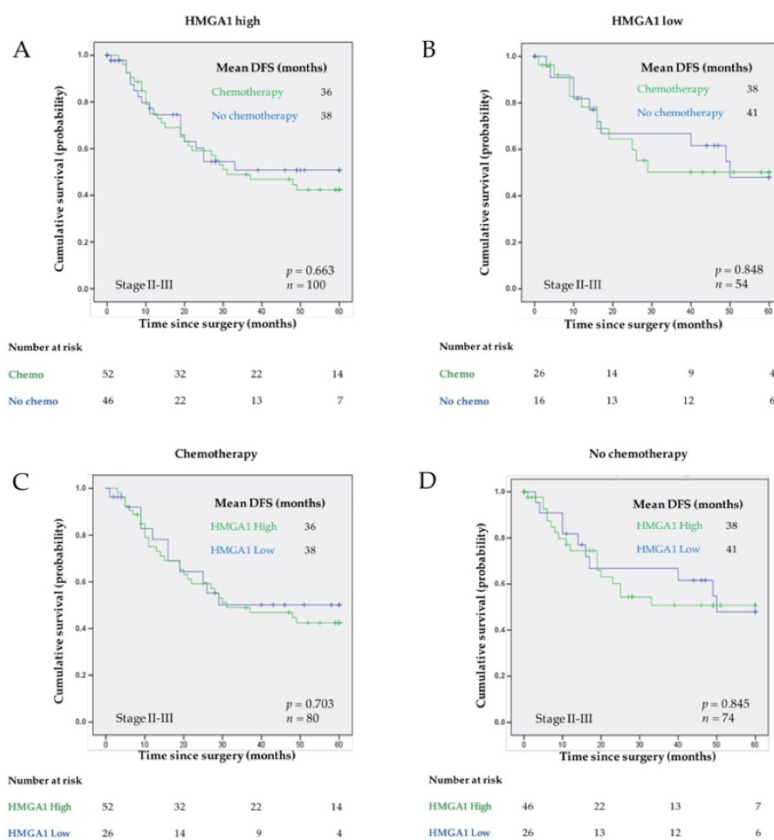


Figure S1. Kaplan-Meier curves showing the probability of DFS according to treatment options in patients having tumors with: (A) high and (B) low expression of HMGA1, respectively. Alternatively, Kaplan-Meier curves showing the probability of DFS according to levels of expression of HMGA1 in patients (C) treated or (D) not treated with adjuvant chemotherapy. Mean DFS is shown for each subgroup of patients. The log-rank test was used to test for differences in DFS between treatment and absence of treatment (A and B) or between high and low levels of HMGA1 (C and D). p value is indicated. The number of patients at risk is specified for 0, 20, 40 and 60 months.

Table S1. Chemotherapy regimens used in the 121 treated GC patients, namely in cases having either high or low expression of HMGA1.

Chemotherapy Regimens			HMGA1 high		HMGA1 low	
Drugs	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Carboplatin and Docetaxel	1	0,9	1	1,3	0	0,0
Carboplatin, 5-Fluorouracil and Taxotere	3	2,6	3	4,0	0	0,0
Cisplatin and 5-Fluorouracil (CF)	9	7,8	7	9,2	2	5,1
Cisplatin, 5-Fluorouracil and Etoposide	2	1,7	1	1,3	1	2,6
Cisplatin, 5-Fluorouracil and Taxotere	26	21,8	14	17,1	12	30,7
Cisplatin, 5-Fluorouracil and Docetaxel (modified DCF)	5	4,3	4	5,3	1	2,6
Cisplatin, Epirubicin and continuous 5-Fluorouracil (ECF)	2	1,7	0	0,0	2	5,1
Cisplatin and Capecitabine (XP)	25	20,9	16	19,7	9	23,1
Cisplatin, Capecitabine and Taxotere	1	0,9	1	1,3	0	0,0
Capecitabine (monotherapy)	6	5,2	4	5,3	2	5,1
Capecitabine, Epirubicin and Oxaliplatin (EOX)	16	11,3	12	13,2	4	7,7
Capecitabine and Oxaliplatin (XELOX)	16	13,9	11	14,5	5	12,9
Capecitabine and Irinotecan	1	0,9	1	1,3	0	0,0
Leucovorin calcium (folinic acid), 5-Fluorouracil and Irinotecan (FOLFIRI)	2	1,7	2	2,6	0	0,0
Leucovorin calcium and 5-Fluorouracil	1	0,9	1	1,3	0	0,0
Leucovorin calcium (folinic acid), 5-Fluorouracil and Oxaliplatin (FOLFOX)	1	0,9	1	1,3	0	0,0
No information	4	2,6	1	1,3	3	5,1
Total	121	100	80	100	41	100

CHAPTER VI

GENERAL DISCUSSION

The relevance of this thesis. Gastric cancer continues to be a serious health problem with high mortality (1). The fact that it is a silent disease in the early stages of its development means that the patients are often diagnosed at a more advanced stage and, therefore, the most effective treatments, namely surgery, are no longer adequate, leaving the patients with limited treatment options such as chemotherapy and/or radiotherapy (2). A common problem with cancer chemotherapy and/or radiotherapy is the development of resistance to the treatment (3). This development of resistance is orchestrated by CSCs which are responsible for promoting the tumorigenic process, being closely involved in the appearance and growth of tumors, in the adaptation and resistance to stresses in the tumor microenvironment, and the reappearance of the disease after an apparently successful treatment (4-8). Therefore, to improve survival from this pathology, several advances in diagnosis and the development of new therapies are needed. Given that CSCs play such a crucial role in tumor development, they must be studied in order to find markers that allow their identification, especially at an early stage of the disease, and/or markers that can be used to exploit their weaknesses, to uncover therapies capable of eliminating these cells. In this thesis we contributed to the study of gastric cancer with results that can help in the identification and elimination of gastric CSCs and with a biomarker that can be used to refer patients to chemotherapy. Briefly, we showed that the expression of the SOX2 can be used to identify gastric CSCs and that it is important for the proliferative activity and resistance to 5-FU presented by these cells. We discovered that gastric CSCs are sensitive to monensin, thus revealing a potential treatment to eliminate these cells. We also revealed that the transcription factor HMGA1 promotes the appearance of gastric CSCs by inducing SOX2 expression. Moreover, we identified that elevated HMGA1 expression in the tumors of patients with gastric cancer can be used as an indicator to recommend chemotherapy to these patients.

The rationale to study CSCs based on the expression of TFs. CSCs have been identified mainly using of cell-surface markers (9). Using the combination of two cell-surface markers (CD34 and CD38), Lapidot et al. identified a small population of CD34⁺/CD38⁻ acute myeloid leukemia cells with stem-like characteristics, such as the ability to initiate the disease after inoculation in immunodeficient mice (10). Since then, the use of cell-surface markers has been applied to identify CSCs in various cancer types, including gastric cancer (11-15). The existence of gastric CSCs was first demonstrated by Takaishi et al. using the CD44 cell-surface marker (15). CD44⁺ cells showed greater resistance to radio- and chemotherapy, ability to form sphere-like structures (gastrospheres) as well as tumorigenic capacity *in vivo*. However, the use of cell-surface markers does not allow monitoring the CSCs in real-time (16). Furthermore, these markers are not specific and vary within the same type of cancer. This is also supported by the study carried out by Takaishi et al. where

they showed that the identification of CSCs using the CD44 marker was only successful in three of six gastric cancer cell lines (15). That is, CD44 allowed the identification of gastric CSCs in MKN-45, MKN-74, and NCI-N87 cell lines, but not in the AGS, Kato III and MKN-28 cell lines (15). Our data also indicated that CD44 cannot be used to mark gastric CSCs in AGS and Kato III cell lines. Furthermore, our results showed that other markers, such as CD133 and ALDH1, are also not suitable for identifying gastric CSCs in AGS and Kato III cell lines. Therefore, ideal CSC markers will be those that are necessary for CSCs to maintain their cancer stem-like characteristics, and that are cross-cutting across multiple cancer types. Thus, TFs are those that stand out most as potential candidates for identifying and studying CSCs (16, 17). TFs are proteins that bind to specific DNA sequences and control gene expression in response to different stimuli and/or growth conditions (18). Several studies have revealed the importance of TFs in the induction and maintenance of stem-like traits. In fact, Takahashi et al. showed that through upregulation of OCT3/4, SOX2, KLF4 and C-MYC they could generate iPS cells from fibroblast cultures (19, 20). Therefore, these TFs have sparked interest in the study of CSCs and several reporter systems based on their expression have been developed and used to identify and study CSCs (21). One of the reporter systems is SORE6-GFP, a lentivirus-based reporter system consisting of six tandem repeats of the SOX2 and OCT4 responsive element that leads to the expression of a GFP (22). Thus, if cells express sufficient levels of SOX2 and/or OCT4 they will become GFP+, allowing the identification and study of CSCs. Through the development and use of the SORE6-GFP system, Tang et al. were able to isolate and characterize breast CSCs (22). Cells marked with this reporter system exhibited self-renewal, asymmetric division, high capacity to initiate tumors and metastases, and resistance to chemotherapy (22). Subsequently, several studies using the SORE6-GFP reporter system were able to successfully identify and characterize CSCs in various cancer types, including sarcoma, malignant mesothelioma, and lymphoma. (23-25). Therefore, as none of the reporter systems have been used in gastric cancer, we decided to use the SORE6-GFP reporter system to identify and characterize gastric CSCs. We chose this reporter system due to the expression of SOX2 in gastric cancer cell lines and to clarify the role of SOX2 in gastric cancer and its impact on gastric CSCs. Furthermore, the identification of CSCs through TFs allows exploring the network of TFs involved in maintaining the cancer stem-like phenotype, thus revealing potential new markers and/or therapeutic targets. For example, in breast cancer, a network has been identified with two or more TFs that induce cancer stem-like cells, such as C-MYC, KLF4, OCT4, NANOG, and SOX2 (26). In gastric CSCs, STAT3 has been targeted by napabucasin. This drug works by suppressing the self-renewal ability of CSCs and induces apoptosis by targeting the STAT3, NANOG and β -catenin signaling pathways (27). Additionally, Nguyen et al. found

that all-trans retinoic acid inhibits tumor growth and targets gastric CSCs by downregulating the expression of CSC markers, such as CD44, ALDH, KLF4 and SOX2 (28). Thus, TFs, in addition to allowing a more specific identification of CSCs, can also be targets of new therapies aimed at them.

The reason we chose AGS, Kato III and SNU638 gastric cancer cell lines. This research work was mainly carried out using gastric cancer cell lines. We chose different cell lines to explore the transversality of the identification and characterization of gastric CSCs, taking SOX2 expression as a starting point, since the SORE6-GFP reporter system is based in SOX2 expression. Therefore, it was important to choose cell lines with SOX2 expression so that we could successfully utilize the SORE6-GFP reporter system. The AGS, Kato III and SNU638 cell lines showed different expression levels of several TFs, including SOX2 and HMGA1, in addition to other molecules. Our results showed that the cells express SOX2 and that it is a heterogeneous expression, that is, within the cell line there are cells that express SOX2 and others with low/no expression of SOX2. The chosen cell lines also have different origins that can impact their characteristics, such as proliferation capacity and drug resistance (in particular, our results revealed that Kato III cells are more drug resistant than AGS). The AGS cell line was originally isolated from fragments of a tumor resected from a female patient with gastric adenocarcinoma who had not received prior therapy (29). The Kato III cell line was established *in vitro* from a pleural effusion (metastatic site) from a male patient with gastric signet ring cell adenocarcinoma (SRCC) (30). The SNU638 cell line was obtained from a metastasis derived from an esophagogastric adenocarcinoma from a male patient (31). Furthermore, the cell lines also have different morphologies. AGS and SNU638 cells have epithelial morphology and grow well adhered to the surface of the flask or plate, while Kato III cells have spherical morphology and grow in two ways in the same cell culture, adherent and in suspension. Additionally, SRCC is a unique subtype of gastric cancer that exhibits impaired cell-cell adhesion (32). Although these gastric cancer cell lines present several differences including different molecular signatures, promoted by the different origin of these cells, which gives them their own characteristics, we were able to identify, isolate and characterize a small population of gastric cancer stem-like cells in the three cell lines using the SORE6-GFP reporter system.

The evidences that led us to consider SORE6+ cells as gastric cancer stem-like cells. Several studies have been carried out to identify and study CSCs in various types of cancer. Characterization of these cells revealed that they shared characteristics of embryonic stem cells. CSCs possess the ability to initiate and sustain tumor growth and development, cellular and metabolic plasticity, self-renewal, asymmetric division, multipotent capacity, radio and chemotherapy resistance, and tumorigenicity *in vivo*. Furthermore, the ability to form sphere-like structures *in vitro* under serum-free and non-

adherent conditions is also considered to be a property of CSCs. Likewise, gastric CSCs exhibit several of the previously mentioned characteristics. Takaishi et al. reported that CSCs were able to form gastrospheres and tumors in nude mice (15). Han et al. work supported these findings by showing that gastric CSCs can form gastrospheres *in vitro* and tumors *in vivo* (33). In addition, they showed that gastric CSCs have self-renewal ability, multipotent capacity and drug resistance, namely 5-FU and cisplatin (33, 34). It was also revealed that CSCs are enriched in TFs involved in promoting and maintaining the stem-like phenotype. There is evidence that overexpression of SOX2, OCT4 and NANOG is crucial for tumor development, tumorigenicity, metastasis, drug resistance and disease recurrence (17). In particular, CSCs identified with the SORE6-GFP reporter system showed enrichment in SOX2, OCT4, C-MYC and NANOG and exhibited the expected characteristics of cancer stem-like cells, such as self-renewal, asymmetric division (generation of heterogeneous progeny), greater tumorsphere formation ability, improved tumor initiation and metastasis *in vivo* and drug resistance (22-25). Therefore, using this novel reporter system, we isolated SORE6+ (CSCs) and SORE6- (non-CSCs) cell subpopulations with differential expression of SOX2. SORE6+ cells were characterized by increased expression of several stemness-associated TFs (SOX2, C-MYC, KLF4, NOTCH1, HIF-1 α and HMGA1), asymmetric division, cell plasticity, multidrug resistance, greater proliferation ability, better gastrosphere formation capacity and greater capacity to form tumors *in vivo* when compared to SORE6- and wt cells. Furthermore, we positively correlated the cancer stem-like features of SORE6+ cells with SOX2 expression. By upregulating SOX2 in SORE6- cells, we observed an increase in cell proliferation and resistance to 5-FU, whereas knockdown of SOX2 in SORE6+ cells decreased cell proliferation and sensitized cells to 5-FU treatment. SOX2 overexpression has been associated with tumor invasion, metastasis and drug resistance. Tian et al. revealed that SOX2 expression increases tumorigenicity and drug resistance of gastric cancer-like cells (35). Moreover, Hutz et al. showed that high levels of SOX2 are involved in gastric cancer cell proliferation, apoptosis and cell cycle regulation, while its suppression decreased cell proliferation and increased apoptosis (36). Therefore, taking into account our results and all the characteristics that have been associated with CSCs and in particular gastric CSCs, we consider that SORE6+ cells are gastric cancer stem-like cells and that SOX2 expression is crucial for the maintenance of the stem-like characteristics of these cells.

The rationale behind using the SORE6-GFP reporter system as a readout of cellular reprogramming toward a stem-like phenotype (in particular, why we consider GFP+ cells as gastric cancer stem-like cells). Understanding what is needed to transform non-CSC cells into CSCs may allow us to discover new targets for eliminating CSCs. SOX2 expression is critical for gastric CSCs, however, it is just the tip of the iceberg and many

other molecules are also associated with the promotion and maintenance of the gastric cancer stem-like phenotype. Thus, we attempted to reprogram gastric non-CSCs into CSCs to find what is required to acquire the CSC phenotype. For this we use SORE6- cells, these cells have the SORE6-GFP reporter system incorporated (guaranteed through selection with puromycin) and are GFP- due to the absence or low expression of SOX2. Since we were able to isolate and characterize a population of gastric cancer cells with cancer stem-like features through the use of the SORE6-GFP reporter system, we could safely assume that when GFP- cells become GFP+ are cancer stem-like cells. It is also important to highlight that SORE6- cells do not express GFP at any point in culture. We have left them in culture for 10 weeks and they never spontaneously acquired GFP expression. So, this indicates that this population is very homogeneous and stable over time. Furthermore, the activation of the reporter system depends on the expression of SOX2 and/or OCT4, therefore, the appearance of GFP+ cells from GFP- cells requires the induction or increase in the expression of one of these TFs (22). In particular, SOX2 expression is essential for the activation of the SORE6-GFP system and the characteristics of gastric CSCs. Thus, we confidently use the SORE6-GFP system as a readout of cellular reprogramming towards a cancer stem-like phenotype.

The use of CPX in cancer treatment and the mechanism behind CPX's ability to convert gastric non-CSCs into cancer stem-like cells. CPX, an iron chelator, is a synthetic antifungal agent commonly used in the treatment of superficial mycoses (37). CPX has a broad-spectrum activity and inhibits most of the clinically relevant dermatophytes, yeasts and molds, and can also act as an antibacterial agent, inhibiting many Gram+ and Gram- bacteria (38, 39). Depending on the dose and treatment duration, CPX can act as a fungistatic or fungicidal (37, 38). Interestingly, CPX's mechanism of action differs from azoles and other antimycotic medications, which may be associated with CPX's ability to chelate iron. Thus, the postulated mechanism of action for CPX includes the chelation of polyvalent metal cations (Fe^{3+} and Al^{3+}), leading to the inhibition of metal-dependent enzymes, such as cytochromes, catalase and peroxidase. In this way, CPX disrupts cellular activities, such as mitochondrial electron transport processes, energy production and nutrient intake through the cell membrane (40). The search for new treatments for cancer has led several studies to explore the effect of CPX on tumor activity. This is because cancer cells require iron to sustain their high rates of proliferation and metabolism and, in particular, the maintenance and expansion of CSCs has been associated with increased intracellular iron accumulation. (41). Thus, CPX's ability to chelate iron made it a potential drug in the treatment of cancer. However, its underlying mechanism remains unknown. Yin et al. reported that CPX inhibited lung cancer cell proliferation, induced DNA damage and reactive oxygen species (ROS) production and suppressed xenograft growth *in vivo* (42).

In hepatocellular cancer, CPX also inhibits cell proliferation and induces the accumulation of ROS. Interestingly, CPX promoted complete autophagic flux (rate of autophagic degradation) in hepatocellular cancer cells, which allowed to attenuate the CPX's antitumor effect (43). Chen et al. reported that CPX inhibits gastric cancer cell proliferation and induces autophagic death by inducing STAT3 ubiquitination (44). Our results support the strong cytotoxic effect of CPX. We showed that treatment of gastric cancer cells with CPX led to reduced cell viability and decreased cell proliferation. However, we observed that under certain treatment conditions, CPX was able to activate the SORE6-GFP reporter system, incorporated into gastric non-CSCs, originating GFP+ cells through upregulation of SOX2 expression in some of the surviving cells. We also observed that the percentage of GFP+ cells was not stable and decreased over time. This decrease may result from loss of GFP expression (asymmetric division and/or GFP+ cells become GFP-, exhibiting cellular plasticity) or from CPX-induced cell death. Furthermore, CPX also upregulated other key stem-related TFs such as KLF4, C-MYC, HIF-1 α , and HMGA1. The increased expression of HIF-1 α led us to consider that CPX's cellular reprogramming effect would be associated with the induction of chemical hypoxia. Liden et al. reported that CPX can induce HIF-1 α stabilization (45). This is relevant because HIF-1 α expression is more expressed in CSCs than in non-CSCs (46, 47) and in fact, our SORE6+ cells express HIF-1 α , unlike SORE6-. Furthermore, Hif-1 α expression was associated with SOX2 expression. To investigate whether the mechanism of CPX to generate CSCs is associated with the HIF-1 α expression/stabilization, we treated SORE6- cells with the hypoxia mimetic agent cobalt chloride (CoCl₂) (48, 49). Treatment led to activation of the SORE6-GFP reporter system, along with increased expression of SOX2, C-MYC, HMGA1 and HIF-1 α . Accordingly, it has been reported that hypoxic conditions can induce CSCs by promoting the expression of key stem-related TFs such as SOX2, OCT4, KLF4, NANOG and Lin28A in glioma, hepatoma and lung cancer (50). However, the molecular mechanism of this dedifferentiation phenomenon under hypoxia needs further studies (50). Our results showed similarities between the effect of CPX and CoCl₂ in promoting CSCs through upregulation of the same set of TFs. Another mechanism that could be involved in the cellular reprogramming capacity of CPX is the induction of cellular senescence, a state that has been associated with stemness (51-53). Herrmann et al. revealed that although CPX inhibits cervical cancer cell proliferation and induces apoptosis, under increased glucose availability it promotes cellular senescence (54). Our results fully support that under some conditions CPX can induce cellular senescence, and the same occurs with the treatment of cells with CoCl₂, revealing more similarities between the mechanisms of the two compounds in the biogenesis of GFP+ cells. However, the mechanism that links senescence and stemness is not completely known and is the subject of many studies and is still a matter of debate. In

fact, it is not known whether it is senescent cells that reprogram or instead if they induce reprogramming of neighboring cells. It is now noticeably clear that senescent cells create an environment prone to cellular reprogramming through the secretion of factors that act on adjacent cells and are known as SASPs (Senescence Associated Secretory Proteins) (53, 55). After CPX treatment, we tried to identify whether the cells with increased beta-gal activity were the ones that gave rise to GFP+ cells, but we were unsuccessful due to technical difficulties in visualizing GFP+ cells and cells with increased beta-gal activity at the same time. Interestingly, CPX treatment inhibited OXPHOS, which can promote senescent cells. These cells display high metabolic activity, preferring energy production from glycolysis rather than OXPHOS, and may better adapt to OXPHOS inhibition caused by CPX treatment (56, 57). Again, the effect of CPX is parallel to the effect of CoCl₂, both activated the SORE6-GFP reporter system in some cells, upregulated the same set of TFs, induced cellular senescence and inhibited OXPHOS. Although we do not know with certainty the mechanism behind CPX-mediated reprogramming of gastric non-CSCs into CSCs, we were able to show that the mechanism is somewhat mimicked by CoCl₂.

The impact of this ability of CPX in the treatment of gastric cancer. As previously mentioned, CPX revealed a potential antitumor effect, and this sparked a lot of interest in repurposing it to cancer treatment. Despite CPX high cytotoxic power against tumor cells, its mechanism of action is not yet fully understood, and this can cause unexpected problems. In addition, there is still very little information about CPX in the clinical setting. The few clinical studies carried out have shown that, at some concentrations, repeated oral administration of CPX has been associated with gastrointestinal toxicity (58). Our results, although supporting the powerful cytotoxic effect of CPX on gastric cancer cells, also recommend caution if its therapeutic use is intended, as it may convert some surviving cells into CSCs. At least at certain concentrations and exposure times, the potential for cellular reprogramming is present through the induction of cellular senescence (which can be reversed), metabolic plasticity, and increased expression of stemness markers (e.g., SOX2, C-MYC, KLF4, HIF-1 α and HMGA1). And as mentioned in Chapter I of this thesis, pre-injury to the stomach can promote gastric cancer if not prevented or treated appropriately. Our data indicated that cells become more resistant to a second CPX exposure, which may be associated with the greater number of cells that become GFP+ with the second treatment compared to the first CPX exposure. Our results also showed that cells that recovered from a first treatment with 8 μ M CPX proliferate more compared to cells that were not subjected to treatment. Regarding CPX resistance, we can only speculate, but it is tempting to say that senescence plays a significant role here, as has been demonstrated for other drugs (59, 60). Therefore, taking our results into account, it is important to think clearly about the use of CPX in the treatment of gastric cancer. Its powerful cytotoxic effect is tempting,

however, its potential to promote the biogenesis of gastric CSCs in certain circumstances may worsen the final outcome of the treatment. The development of resistance to CPX is also an obstacle that must be taken into account when considering the use of this drug in cancer chemotherapy. Thus, several factors must be considered for the safe and efficient use of CPX in the treatment of stomach cancer.

The influence of HMGA1 expression on the reprogramming of gastric non-CSCs into cancer stem-like cells and the relevance of its expression in gastric cancer. One of the TFs that also appeared upregulated in CPX-treated cells was HMGA1. When we looked at its expression in SORE6 cells, we found that SORE6⁺ cells are also enriched in HMGA1 compared to SORE6⁻ cells. Thus, since HMGA1 has been shown to have an active role in promoting stemness (61-64), we decided to evaluate the effect of HMGA1 overexpression in SORE6⁻ cells. Our results showed HMGA1 overexpression managed to activate the SORE6-GFP reporter system in some cells, and that this activation resulted from the induction of SOX2 expression. We then sorted the cells to compare cells that remained GFP⁻ with those that became GFP⁺. HMGA1 GFP⁺ cells were found to be enriched in SOX2, C-MYC and KLF4 compared to HMGA1 GFP⁻. SNU638 HMGA1 GFP⁺ were also enriched in OCT4. Studies have demonstrated that HMGA1 regulates and promotes SOX2 expression (65, 66). HMGA1 has also been reported to induce C-MYC expression and appears to directly regulate KLF4 expression (63, 67). Overexpression of HMGA1 in ovarian cancer cells increased several stemness-related genes, including KLF4 (63). Induction of OCT4 expression has also been linked to HMGA1 expression (66, 68). Regarding HMGA1 expression, it was expected that it would not be significantly different between both cell populations, as HMGA1 overexpression was done using DOX-inducible lentivirus vectors and after transduction and treatment with DOX the cells were selected with zeocin to ensure that all cells tested had the HMGA1 expression vector incorporated. The reason why HMGA1 overexpression only activated the SORE6-GFP reporter system in some cells may be associated with the low efficiency of cellular reprogramming using TFs. HMGA1 GFP⁺ cells exhibited cancer stem-like characteristics such as greater proliferative activity, greater ability to form colonies and greater resistance to 5-FU compared to HMGA1 GFP⁻. However, the lack of significant differences in HMGA1 expression between HMGA1 GFP⁺ and HMGA1 GFP⁻ cells suggests that it is likely not HMGA1 expression that is associated with these features of HMGA1 GFP⁺, but rather SOX2, whose expression we demonstrated to directly impact cell proliferation and resistance to 5-FU. Since HMGA1 enhances reprogramming of somatic cells to iPS cells (68), we decided to treat HMGA1 GFP⁺ and HMGA1 GFP⁻ cells with CPX to see if the efficiency of cellular reprogramming from GFP⁻ to GFP⁺ would increase. Our data clearly showed that overexpression of HMGA1 increases the ability of CPX to reprogram GFP⁻

cells into GFP+. To understand the clinical value of HMGA1 expression in gastric cancer, we observed the nuclear expression of HMGA1 by immunohistochemistry in a series of 323 gastric tumor samples (stages I to IV), the largest series of tumors used to date for this purpose and the most complete in terms of clinicopathological features, survival data, and treatment data. Although we did not find a significant correlation between HMGA1 and SOX2 we disclose that HMGA1 expression as a useful marker to predict response to chemotherapy in gastric cancer. We uncover that patients with high levels of HMGA1 displayed significantly better overall survival when they were treated with chemotherapy, compared to the non-treated ones.

How monensin targets gastric cancer stem-like cells and can be used to treat gastric cancer. The recognition of CSCs as responsible for the tumorigenic process and acquisition of resistance to therapy has made them attractive targets for cancer treatment. To search for compounds capable of selectively targeting SORE6+ cells, we performed a high-throughput small molecule screening. Our results revealed that the small molecule monensin can specifically eliminate SORE6+ cells. This effect becomes even clearer when we use the already heterogeneous population of SORE6+ cells, containing GFP+ and GFP- cells, resulting from several asymmetric divisions and cell plasticity. These results are interesting because monensin belongs to the same class as salinomycin, a very promising drug for the specific elimination of CSCs, which may allow drawing parallels between the mechanism of action involved in targeting CSCs. Gupta et al. reported that salinomycin was able to reduce the proportion of CSCs by >100-fold (69). They also showed that salinomycin treatment suppresses the expression of breast CSC markers, inhibits tumor growth *in vivo* and induces epithelial differentiation of tumor cells (69). Salinomycin appears to target CSCs by interfering with mitochondrial functions and several signaling pathways, such as Notch, Wnt/ β -catenin, Hedgehog and Akt (70, 71). Although the mechanism of action of monensin responsible for this ability is not fully known, several studies have contributed with information about the antitumor effect of monensin. Ketola et al. showed that monensin is a well-tolerated drug with cancer-specific activity, inducing apoptosis, increasing intracellular oxidative stress and reducing androgen receptor in prostate cancer cells (72). Later, Yao et al. reported that monensin induces inhibition of ovarian cancer cells proliferation, invasion, migration and xenograft tumor growth (73). Urbaniak et al. through the use of an organoid model of glioblastoma showed that glioblastoma is very sensitive to monensin. They also discovered that monensin is capable of causing DNA fragmentation, decreasing cell migration, inducing apoptosis and autophagy (74). Xin et al. suggested that the antitumor effect of monensin in melanoma possibly arises from monensin's ability to inhibit the production of stem-like characteristics of melanoma stem cells, somewhat promoting the differentiation of these cells (75). Wang et al. showed possible mechanisms

involved in the antitumor effect of monensin in chemotherapy-resistant pancreatic cancer cells. Monensin inhibited cell proliferation and migration by suppressing EGFR expression and the E2F/DP1, STAT1/2, NFkB, AP-1, Elk-1/SRF pathways (76). Zhou et al. showed that in colorectal cancer cells, monensin also induces apoptosis, inhibits cell proliferation, migration and cell cycle progression. They propose that the antitumor effect of monensin occurs through targeting the IGF1R signaling pathway (77). Recently, Clemente et al. showed that monensin can selectively kill hepatocellular carcinoma cells by increasing their intracellular Na⁺ levels (78). Additionally, monensin leads to a depletion of intracellular ATP and cell damage phenotype (78). Therefore, it is important to continue investigating the tumor effect of monensin and the mechanism behind this effect, as its effect appears promising and selective in several types of cancer. Thus, in gastric cancer it may represent a new therapy that in more advanced stages can be combined with other drugs to reduce or block tumor growth.

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CHAPTER VII

CONCLUDING REMARKS

Altogether, our studies revealed that we can effectively isolate and characterize gastric CSCs based on the stem-related TFs expression. Our results also indicated that there is a network of TFs responsible for the characteristics of gastric CSC orchestrated by SOX2. We successfully reprogrammed non-CSCs into CSCs using CPX, CoCl₂ or a HMGA1 DOX-inducible lentiviral vector through inducing SOX2 expression. The mechanism of action of CPX (responsible for the appearance of CSCs) appears to be comparable to that of CoCl₂ and seems to be associated with an increase of SOX2, C-MYC, HMGA1, KLF4 and HIF-1 α expression. Interestingly, treatment with CPX or CoCl₂ also leads to cellular senescence, which has been linked to CSCs properties. Therefore, CPX may be a useful tool for obtaining gastric cancer stem-like cells from non-CSCs, particularly when cells previously treated with CPX are re-exposed to the compound. Our results also draw attention to the powerful cytotoxic effect of CPX on gastric cancer cells, however caution is recommended if its therapeutic use is intended, as it can kill most tumor cells but can also convert surviving cells into CSCs. Furthermore, our studies also revealed that monensin can selectively target gastric CSCs, which may represent a worthy therapeutic weapon against gastric cancer. Finally, we uncovered that gastric cancer patients with high levels of HMGA1 expression in the tumor benefit more from chemotherapy treatment than those with low levels of this protein. Overall, this thesis contributed with new information to a better understanding of the characteristics, behavior and impact of gastric CSCs on the development and treatment of gastric cancer. It also presented new gastric CSCs biomarkers and potential therapeutic agents.

APPENDIX

This section contains the following publication:

Pádua, D., Figueira, P., Ribeiro, I., Almeida, R., & Mesquita, P. (2020). *The Relevance of Transcription Factors in Gastric and Colorectal Cancer Stem Cells Identification and Eradication*. *Frontiers in cell and developmental biology*, 8, 442. <https://doi.org/10.3389/fcell.2020.00442>



The Relevance of Transcription Factors in Gastric and Colorectal Cancer Stem Cells Identification and Eradication

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Stem Cell Research,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 20 March 2020

Accepted: 11 May 2020

Published: 18 June 2020

Citation:

Pádua D, Figueira P, Ribeiro I,
Almeida R and Mesquita P (2020) The
Relevance of Transcription Factors
in Gastric and Colorectal Cancer
Stem Cells Identification
and Eradication.
Front. Cell Dev. Biol. 8:442.
doi: 10.3389/fcell.2020.00442

Gastric and colorectal cancers have a high incidence and mortality worldwide. The presence of cancer stem cells (CSCs) within the tumor mass has been indicated as the main reason for tumor relapse, metastasis and therapy resistance, leading to poor overall survival. Thus, the elimination of CSCs became a crucial goal for cancer treatment. The identification of these cells has been performed by using cell-surface markers, a reliable approach, however it lacks specificity and usually differs among tumor type and in some cases even within the same type. In theory, the ideal CSC markers are those that are required to maintain their stemness features. The knowledge that CSCs exhibit characteristics comparable to normal stem cells that could be associated with the expression of similar transcription factors (TFs) including SOX2, OCT4, NANOG, KLF4 and c-Myc, and signaling pathways such as the Wnt/ β -catenin, Hedgehog (Hh), Notch and PI3K/AKT/mTOR directed the attention to the use of these similarities to identify and target CSCs in different tumor types. Several studies have demonstrated that the abnormal expression of some TFs and the dysregulation of signaling pathways are associated with tumorigenesis and CSC phenotype. The disclosure of common and appropriate biomarkers for CSCs will provide an incredible tool for cancer prognosis and treatment. Therefore, this review aims to gather the new insights in gastric and colorectal CSC identification specially by using TFs as biomarkers and divulge promising drugs that have been found and tested for targeting these cells.

Keywords: gastric cancer, colorectal cancer, transcription factors, cancer stem cells, signaling pathways, targeted therapy

INTRODUCTION

Gastrointestinal malignancies are listed among the major causes of cancer death worldwide, being associated with environmental and genetic risk factors such as older age, chronic inflammation, family history, smoking, dietary patterns, overweight and physical inactivity, as well as gut microbiota (Lochhead and El-Omar, 2008; Mattiuzzi et al., 2019). Gastric cancer (GC) and colorectal cancer (CRC) are among the top five most incident and deadly cancers worldwide

(Bray et al., 2018). As surgical techniques improve, as well as radiotherapy, chemotherapy and neoadjuvant therapies, the 5-year survival rate can reach up to 95% for early GC and 90% for localized CRC (Song et al., 2017; Sonbol et al., 2019). However, most patients have advanced-stage disease at diagnosis and so the best surgical window is missed (Song et al., 2017; Bray et al., 2018). They develop recurrent loco-regional disease or distant metastases with consequent decrease in survival. The heterogeneity of cancer at molecular, histological and phenotypic levels plays an important role in therapy resistance and tumor recurrence, being cancer stem cells (CSCs) among the major causative factors of cancer heterogeneity and treatment failure (Iseghohi, 2016; Gullo et al., 2018). The CSC model of tumor progression hypothesizes that a small subpopulation of cancer cells that display stem-like properties sustains tumor growth, metastasis, relapse and resistance to chemotherapy (Iseghohi, 2016). CSCs can undergo symmetric and asymmetric divisions, having the ability to give rise to all the different types of cancer cells within the tumor (Marjanovic et al., 2013). The origin of CSCs is still unclear and controversial (Dalerba et al., 2007a; Brunner et al., 2012). Various hypotheses suggest that depending on the tumor type, CSCs might be derived from adult stem cells, adult progenitor cells that underwent mutations, or differentiated cells that gained stem-like properties through dedifferentiation (Basu et al., 2016; Phi et al., 2018). A large number of studies demonstrate that CSCs share biomarkers with normal stem cells, thus specific markers for their identification have been explored in recent years. It is known that some transcription factors (TFs) can be re-expressed or reactivated in CSCs, playing a crucial role in the reprogramming of these cells. This review aims to provide a better understanding on how TFs associated with gastric and colorectal CSCs phenotype can be used for CSCs identification, characterization and targeted therapy.

THE CHALLENGES OF CANCER STEM CELL IDENTIFICATION

The identification of normal stem cells is now an easy process due to their well-recognized set of biomarkers whereas the identification of CSCs is a challenging task resulting from their complex phenotype, that differs from one tumor to another (Pattabiraman and Weinberg, 2014). Additionally, CSCs represent a very small percentage of tumor cells within the total tumor mass making even harder their detection in heterogeneous tumors (Kim Y. et al., 2009). The use of different combinations of cell-surface markers has been the main strategy to identify CSCs in several tumor types (Magee et al., 2012). The cell-surface markers are chosen according to their expression and relevance in the tumor type allowing the separation of CSCs from non-CSCs (Pattabiraman and Weinberg, 2014). In GC and CRC, the list of cell-surface markers capable of identifying CSCs is growing (Table 1). This means that the interest in gastric and colorectal CSCs identification and targeting is rising but also means that the already used markers are not uniformly advantageous for CSCs detection. In particular, inconsistencies remain concerning which cell-surface marker may be the ideal marker to identify gastric

and colorectal CSCs from cell lines and primary tumors (Takaishi et al., 2009; Zhang et al., 2011; Jiang Y. et al., 2012; Rocco et al., 2012; Wakamatsu et al., 2012; Brungs et al., 2016).

Gastric Cancer

Several cell-surface markers have emerged for gastric CSC identification. The transmembrane glycoprotein CD44 was the first described cell-surface marker used in gastric CSC identification. Takaishi et al. (2009) and his collaborators analyzed a panel of six GC cell lines and in three of them – NCI-N87, MKN-45 and MKN-74 – a small CD44⁺ cell subpopulation displayed CSC features such as self-renewal, asymmetric division, spheroid colony formation, and *in vivo* tumorigenic ability. They also observed that the CD44⁺ subpopulation had a higher resistance to anticancer drugs when compared to CD44[−] cells (Takaishi et al., 2009). However, in the other three cell lines – AGS, Kato III and MKN28 – the CD44 cell-surface marker was not able to mark cells with stem cell properties (Takaishi et al., 2009). Clinically, CD44⁺ cancer cells at the invasive GC front are associated with poor patient survival (Nosrati et al., 2014; Kodama et al., 2017). Later, Zhang et al. (2011) combined CD44 with CD24, a signal transducer, and successfully detected a CD44⁺CD24⁺ cellular subpopulation with CSCs characteristics, such as the capability to self-renew and to originate differentiated progeny (Zhang et al., 2011). Additionally, they showed that CD44⁺CD24⁺ cells had higher ability to form tumors when injected into immunodeficient mice, compared to the CD44[−]CD24[−] cells (Zhang et al., 2011). The CD54 cell-surface marker, also known as ICAM-1 (intercellular adhesion molecule 1), was combined with CD44 to isolate gastric CSCs from tumor tissues and peripheral blood of patients with GC (Chen et al., 2012). The CD44⁺CD54⁺ cells exhibited *in vitro* and *in vivo* self-renewal ability, formed gastric tumorspheres and originated tumors similar to the original human tumor when injected into immunodeficient mice (Chen et al., 2012). The epithelial cell adhesion molecule (Epcam) has also been used in combination with CD44 to mark gastric CSCs. The small Epcam⁺/CD44⁺ subpopulation isolated from primary human GC tissues was more resistant to anticancer drugs including 5-fluorouracil (5-FU), doxorubicin, vinblastine and paclitaxel, when compared with Epcam⁺/CD44[−], Epcam[−]/CD44⁺ and Epcam[−]/CD44[−] cells (Brabletz et al., 2005; Han et al., 2011). It also showed capacity to form sphere-like structures in serum free conditions and greater ability to originate tumors in immunocompromised mice (Han et al., 2011). The tumors formed after inoculation of the Epcam⁺/CD44⁺ cells recapitulated the heterogeneous morphology and phenotype present in the original gastric tumor (Han et al., 2011). Moreover, Fukamachi et al. (2013) identified another potential gastric CSC marker, the CD49f, an integrin $\alpha 6$ (ITGA6) that is a subunit of laminin receptors. Their work showed that CD49f⁺ cells from GC originated tumors when subcutaneously injected into immunodeficient mice, while CD49f[−] cells did not (Fukamachi et al., 2013). They also demonstrated that some of the CD49f⁺ sphere-forming cells were more resistant to doxorubicin, 5-FU and doxifluridine than the other GC cells studied (Fukamachi et al., 2013). Another cell-surface marker identified as a gastric

TABLE 1 | Cancer stem cell (CSC) biomarkers for gastric and colorectal cancer.

CSCs biomarkers	Gastric Cancer	Colorectal Cancer
	References	References
CD44	Takaishi et al., 2009; Zhang X. et al., 2016	Du et al., 2008; Huang et al., 2015
CD44 combined with CD24	Zhang et al., 2011	—
CD44 combined with CD54	Chen et al., 2012	—
CD44 combined with EpCAM	Han et al., 2011	Dalerba et al., 2007b
CD44 combined with CD133	—	Huang et al., 2015
CD49f	Fukamachi et al., 2013	Haraguchi et al., 2013
CD71	Ohkuma et al., 2012	—
CD90	Jiang J. et al., 2012	—
CD133	Fukamachi et al., 2011; Zhang X. et al., 2016	O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Huang et al., 2015
CD133 combined with CD166	—	Dalerba et al., 2007b
CD184 (CXCR4)	Fujita et al., 2015	—
CD200	—	Zhang S.S. et al., 2016
Lgr5	Gong et al., 2016	—
Lgr5 combined with EpCAM	—	Kemper et al., 2012
Lgr5 combined with EpCAM and CD44	—	Leng et al., 2018
ALDH	Zhi et al., 2011	Huang et al., 2009
SOX2	Pádua et al., 2020	Takeda et al., 2018

The table contains some of the most reported CSCs markers used for both tumor types.

CSC marker is the CD71 transferrin receptor. In this case, it was demonstrated that the CD71[−] subpopulation from the MKN-1 GC cell line displayed CSC features, contrary to CD71⁺ cells. The CD71[−] cells were more resistant to 5-FU than CD71⁺, had higher tumorigenic ability and were mostly present in the invasive front of the tumor (Ohkuma et al., 2012). The cell-surface glycoprotein CD90 (Thy-1) appeared as a potential gastric CSC marker since it was capable of identifying a small population with *in vivo* tumorigenic and self-renewal ability (Jiang J. et al., 2012). Additionally, 25% of the gastric primary tumors possessed higher expression of erb-b2 receptor tyrosine kinase 2 (HER2), which was correlated with the higher expression of CD90 (Jiang J. et al., 2012). CD133 (prominin-1), a pentaspan transmembrane glycoprotein, is described as a gastric CSC marker due to the fact that its expression is positively correlated with tumor aggressiveness in GC patients (Fukamachi et al., 2011; Lee et al., 2012; Wakamatsu et al., 2012; Hashimoto et al., 2014; Nosrati et al., 2014). Zhao et al. showed that the frequency of CD133⁺ in gastric primary tumors samples was higher than CD133[−] cells and CD133 was associated with poor prognosis in GC (Zhao et al., 2010). Also, spheroid cells from GC cell lines and primary GC tissues presented CD133 expression and displayed several features of CSCs (Zhang X. et al., 2016). New cell-surface markers have emerged in the study of gastric CSCs and demonstrated to be able to mark a small population in GC with stem-like features, specifically Lgr5 (leucine-rich repeat-containing G-protein coupled receptor 5) and CXCR4 (C-X-C chemokine receptor type 4) also known as CD184 (Fujita et al., 2015; Gong et al., 2016). Also, the intracellular enzyme aldehyde dehydrogenase (ALDH) has been used to identify gastric CSCs (Zhi et al., 2011; Wakamatsu et al., 2012). Zhi et al. (2011) were able to divide NCI-N87 and SNU-1 GC cell lines into ALDH⁺ and ALDH[−] cells.

The ALDH⁺ cells presented CSC features such as higher levels of SOX2, NANOG and Nestin, formed more sphere-like structures and had higher resistance to 5-FU and cisplatin (Zhi et al., 2011). They also showed that ALDH⁺ cells were more sensitive to salinomycin, a drug proposed to target CSCs (Zhi et al., 2011).

Colorectal Cancer

Several specific cell-surface markers were used to identify colorectal CSCs (Todaro et al., 2010; Vaiopoulos et al., 2012; Rassouli et al., 2016; Wahab et al., 2017; Zhou et al., 2017; Boesch et al., 2018; Munro et al., 2018; Parizadeh et al., 2019; van der Heijden and Vermeulen, 2019). CD133 was shown to be a robust CSC-surface marker in CRC (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Akbari et al., 2020). Positive expression of CD133 was, for the first time, associated with a significantly worse survival and poorer clinical response to 5-FU-based chemotherapy in CRC patients (Ong et al., 2010). CD44 is also a valid marker of colorectal CSCs (Du et al., 2008). In combination with EpCAM it has already been considered a better strategy, when compared to CD133 (Dalerba et al., 2007b). Furthermore, CD166 (another transmembrane glycoprotein) could be used to co-purify CSCs (Dalerba et al., 2007b). More recently, colonospheres and chemoresistant CRC cells were found to be enriched with CD133 and CD44 (Huang et al., 2015). A strategy that combines CD133 with CD44, seems to be more reliable to isolate colorectal CSCs from both cell lines and primary tumors (Abbasian et al., 2019). Horst et al. (2009) showed that CD133 may have a better prognostic capacity *per se*, but the combination of CD133, CD44, and CD166 markers may stratify better the risk of CRC. Lgr5 positivity identifies human colorectal CSCs and is a prognostic factor for CRC (Kemper et al., 2012; Jiang Y. et al., 2016). Lgr5 overexpression has been shown to be important in

CRC progression, as a result of its role in potentiating the Wnt signaling pathway (Carmon et al., 2012). de Sousa e Melo et al. (2017) have shown that Lgr5⁺ CSCs are crucial for the formation of liver metastases, while in the primary tumor Lgr5⁻ cells proliferate and are capable of originating Lgr5⁺ cells, assuring a rapid tumor growth when treatment ends. Lgr5 combined with CD44 and EpCAM can further improve the identification of CSCs in CRC (Leng et al., 2018). Vermeulen et al. (2008) have shown that spheroid cultures of colon CSCs express CD133, CD166, CD44, CD29, CD24, Lgr5, and nuclear β -catenin, all of which described markers of the CSC population. Also, CD200 showed a role as a CSC marker in colon cancer, and CD49f was associated with tumor cell invasion and metastasis in colon cancer working as an important marker for identifying colorectal CSCs (Robertson et al., 2009; Haraguchi et al., 2013; Zhang S.S. et al., 2016). ALDH1 also appears as a specific marker for colonic CSCs and as an independent prognostic factor for patients with CRC (Huang et al., 2009; Lugli et al., 2010; Kahlert et al., 2012; Goossens-Beumer et al., 2014; Zhou et al., 2017; Munro et al., 2018). In patients with stage II-III rectal cancer that received radio-chemotherapy, ALDH1 expression was correlated with poor prognosis, cancer relapse and metastasis (Huang et al., 2009; Deng et al., 2014).

TRANSCRIPTION FACTORS AS POTENTIAL CSC MARKERS

One of the challenges to target CSCs is to identify specific markers as well as to uncover targetable molecular features associated with their phenotype. It is known that normal stem cells and CSCs share core stemness signaling pathways such as Notch, Hedgehog, WNT/ β -catenin and JAK/STAT, that have pivotal roles in maintaining stem cell properties and regulating their transcriptional program (Chen K. et al., 2013). SOX2, OCT4, KLF4, and NANOG are some of the key TFs known to promote stemness by upregulating genes involved in self-renewal and pluripotency, while suppressing genes involved in differentiation (Young, 2011; Tang et al., 2015; Buczek et al., 2018). Key stem cell TFs like SOX2, OCT4, and NANOG have been proven to be overexpressed in CSCs. For that reason, fluorescence reporter systems driven by portions of promoters where these proteins bind were developed to allow CSCs to be labeled and tracked in various types of cancer. These reporter systems seem to be a powerful tool to identify and study CSCs more efficiently than cell-surface markers (Saygin et al., 2016). Tang et al. (2015) developed a flexible lentiviral-based reporter system (SORE6-GFP) that allows direct visualization of CSCs based on SOX2 and OCT4 expression. By using this novel reporter system, our group was able to isolate gastric CSCs from two phenotypically different GC cell lines (AGS and Kato III) (Pádua et al., 2020). Using the same principle, other authors have been developing similar systems to identify and characterize CSCs in a variety of solid tumors. Buczek et al. (2018) established a method using a lentiviral construct carrying the promoter of NANOG to identify prostate CSCs and more recently, Ghanei et al. (2020) introduced a similar approach

for the isolation of CSCs in a breast cancer cell line based on the single expression of OCT4. Although the use of cell-surface markers is the most trusted approach to detect these cells, several studies demonstrated that they lack specificity and cannot be used for real time assessment of CSCs behavior, which could give new insights about their properties and possible targets (Tang et al., 2015). Growing evidences support that specific TFs overexpressed in normal gastrointestinal stem cells may contribute to the self-renewal characteristics of CSCs in GC and CRC and are related with patient prognosis (Hadjimichael et al., 2015; Zhao et al., 2017). This makes them a powerful tool in CSCs identification and study.

SOX2 (SRY-Box Transcription Factor 2)

SOX2 is a master regulator that belongs to the family of high-mobility group TFs. It plays many roles throughout development and cell differentiation in normal tissues, namely in mammalian embryogenesis, morphogenesis and homeostasis of the foregut-derived epithelia of the esophagus, lung and trachea (Avilion et al., 2003; Sarkar and Hochedlinger, 2013). SOX2 role in stemness was strengthened with Takahashi and Yamanaka's findings when reprogramming mouse embryonic fibroblasts into induced pluripotent stem (iPS) cells, by introducing SOX2 along with OCT3/4, c-Myc and KLF4 (Takahashi and Yamanaka, 2006). In cancer, SOX2 has increasingly been associated with a CSC phenotype in several tumors (Wuebben and Rizzino, 2017; Takeda et al., 2018). In GC, SOX2 role is still controversial: some authors associate its high expression with a more aggressive phenotype, poor prognosis and worse response to therapy whereas others have shown the opposite (Hütz et al., 2013; Camilo et al., 2014; Carrasco-Garcia et al., 2016; Zhang X. et al., 2016; Wuebben and Rizzino, 2017; Basati et al., 2020; Pádua et al., 2020). Our group identified subpopulations of gastric CSCs in two human cell lines based on the expression of SOX2 and showed that SOX2⁺ cells presented CSCs properties, including higher proliferation and ability to form gastrospheres, enhanced *in vivo* tumorigenesis and increased resistance to 5-FU (Pádua et al., 2020). Additionally, Hütz et al. (2013) observed that inhibition of SOX2 resulted in reduced cell proliferation and migration, increased apoptosis, changes in cell cycle and reduced tumorigenic potential of cells *in vivo*. Similar results were observed *in vivo*, where suppression of SOX2 resulted in reduced tumor growth and decreased tumorigenicity (Tian et al., 2012; Hütz et al., 2013). In CRC, SOX2 overexpression has been associated with tumor progression and disease recurrence and SOX2 *de novo* expression was associated with poorly differentiated and more invasive tumors and poor patient overall survival (Lundberg et al., 2014; Lundberg et al., 2016). Takeda et al. (2018) have shown that SOX2⁺ cells developed chemoresistance to oxaliplatin and 5-FU, exhibiting typical asymmetric cell division and higher CSC markers expression. They concluded that colon cancer cells expressing SOX2 behave like CSCs and are therefore associated with poor prognosis (Lundberg et al., 2016; Takeda et al., 2018). Taken together, these findings indicate that SOX2 has a critical role in several aspects of CSCs biology.

OCT4 (POU Class 5 Homeobox 1)

OCT4, a homeodomain TF of the Pit-Oct-Unc family, is expressed in embryonic stem cells, where it maintains stem cell-like properties, and in adult stem cells, being involved in their proliferation and differentiation (Loh et al., 2006; Han et al., 2014). Abnormal expression of OCT4 has been observed in different tumor types, including GC and CRC (Dai et al., 2013; Basati et al., 2020). Studies report that its expression is positively correlated with more aggressive and metastatic tumors, as well as with poorer overall prognosis (Zhang X. et al., 2016; Basati et al., 2020). Chen and collaborators demonstrated that overexpression of OCT4 in GC cells led differentiated cancer cells to become undifferentiated and acquiring self-renewal capacity (Chen et al., 2009) and another study reported that downregulation of OCT4 induced differentiation in GC cells (Tai et al., 2005). Additionally, Zhang X. et al. (2016) demonstrated a p-ERK mediated positive feedback loop between the cell surface marker CD44 and OCT4, responsible for sustaining gastric CSCs properties. OCT4 has also been reported in colon CSCs and its knockdown inhibits cell migration and invasion, suggesting it may act as a novel prognostic marker in CRC (Miyoshi et al., 2010, 2018; Dai et al., 2013; Amini et al., 2014).

KLF4 (Kruppel Like Factor 4)

KLF4, also known as the gut-enriched Kruppel like factor (GKLF), is strongly expressed in post-mitotic and terminally differentiated epithelial tissues, along with those of the gastrointestinal tract (Cho et al., 2007; Cui et al., 2013). It is suggested that it may have an anticancer role in GC, being downregulated due to hypermethylation and loss of heterozygosity in gastric CSCs (Cho et al., 2007). Several studies have shown that KLF4 low expression is negatively associated with patient overall survival and may be a useful prognostic marker in GC patients (Li et al., 2012; Zhang et al., 2012; Hsu et al., 2013; Hashimoto et al., 2017; Zhao et al., 2020). Regarding CRC, KLF4 role is still not clear. Some studies reveal KLF4 is overexpressed in colon CSCs and its knockdown affects the stemness phenotype and decreases the cells malignant profile, while others demonstrate that loss of expression is associated with stem-like features namely formation of colonospheres, cell growth arrest, uncontrolled cell proliferation, pluripotency and self-renewal (Shie et al., 2000; Wei et al., 2006; Leng et al., 2013; Hadjimichael et al., 2015).

NANOG (Nanog Homeobox)

NANOG was first discovered in embryonic stem cells (ESCs) and is a key TF involved in self-renewal and multipotency (Chambers et al., 2003). It is typically silenced in normal somatic cells, though abnormal expression has been reported in malignant tumors, such as GC and CRC (Lin et al., 2012; Hadjimichael et al., 2015). Previous studies demonstrate that NANOG is highly expressed in GC and significantly associated with tumor size and grade, along with decreased overall survival (Iv Santaliz-Ruiz et al., 2014; Basati et al., 2020). Although studies indicate NANOG is associated with a CSC phenotype, it remains unclear its role in CSC maintenance in GC (Iv Santaliz-Ruiz et al.,

2014). With respect to CRC, NANOG overexpression has been associated with colony formation and stem cell properties, as well as worse prognosis (Ibrahim et al., 2012; Zhang et al., 2013; Hadjimichael et al., 2015).

c-Myc (MYC Proto-Oncogene, bHLH Transcription Factor)

c-Myc is an essential TF that regulates genes that take part in biological processes such as self-renewal, differentiation, growth and metabolism (Dang, 2013; Bretones et al., 2015). Although it is one of the most commonly activated oncogenes involved in the pathogenesis of cancer, its overexpression alone is unable to induce the transformation of normal cells into tumor cells (Yang et al., 2020). The role of c-Myc in GC is less studied than in other tumor types. It has been suggested as a CSC marker in some tumors such as small-cell lung cancer, prostate cancer, neuroblastoma, glioblastoma and hematopoietic malignancies, but the expression and relevance in GC has not yet been clarified (Yang et al., 2020). Some authors associate c-Myc deregulation with poor prognostic features (Han et al., 1999; de Souza et al., 2013; Wang et al., 2016). Upregulation of c-Myc is common in 70% of CRC cases and it has been shown to have a crucial role in maintaining chemoresistance and self-renewal, being overexpressed in colon CSCs (Muzny et al., 2012; Zhang et al., 2019). Despite some controversial results, it has been shown that high expression of c-Myc is an independent poor prognostic factor in CRC (Lee et al., 2015; Wang et al., 2017).

SOX9 (SRY-Box Transcription Factor 9)

SOX9 regulates developmental processes such as male sex determination, chondrogenesis, neurogenesis, and neural crest development (Jo et al., 2014). Also, it plays a vital role in cell fate decisions and stem cell maintenance during embryonic development and adulthood in several organs, including the gastrointestinal tract (Bastide et al., 2007; Huch and Clevers, 2011). Its role in GC is still conflicting, while some studies defend an association between lower survival and SOX9 high expression, others demonstrate poor prognosis with a decreased level of SOX9 expression (Sun et al., 2012; Santos et al., 2016; Mesquita et al., 2019). Also, both oncogenic and tumor suppressor activity of SOX9 have been implicated in CRC (Darido et al., 2008; Lu et al., 2008; Matheu et al., 2012; Prévostel and Blache, 2017). Previous studies suggest that this TF can influence tumor proliferation and progression, mainly through the regulation of the CSC pool, and could correlate with poor prognosis (Lu et al., 2008; Espersen et al., 2015; Javier et al., 2016).

GLI1 (GLI Family Zinc Finger 1)

GLI1 is part of the Sonic Hedgehog (SHH) pathway and seems to be essential for the maintenance of cancer cells with stem like properties in both GC and CRC (Zhang X. et al., 2016; Yang et al., 2018). In GC, its expression is significantly higher in metastatic cancer tissues and is positively correlated with a more aggressive tumor phenotype (Zhang X. et al., 2016). Furthermore, it has been observed that GLI1 overexpression promotes a CSC phenotype enhancing cell proliferation, migration and therapy

resistance (Dong et al., 2019; Yao et al., 2019). GLI1 also plays an important role in CSC characteristics related with aggressiveness and metastatic spread of CRC cells leading to decreased survival. Furthermore, GLI1 knockdown downregulates CD133/SOX9 expression and clonogenic ability of CRC cells, indicating this TF could be a potential marker for CSCs in CRC (Yang et al., 2018).

STAT3 (Signal Transducer and Activator of Transcription 3)

STAT3 plays an important role in the regulation of various physiological functions, such as inflammation, proliferation and invasion, being highly expressed in gastric and colorectal CSCs (Yu H. et al., 2009; Lin et al., 2011; Hajimoradi et al., 2016; Sonbol et al., 2019). In gastric CSCs, high expression of STAT3 has been reported to be involved in stemness properties and invasive ability (Hajimoradi et al., 2016; Jiang et al., 2017; Sonbol et al., 2019). Regarding its prognostic value several studies report STAT3 activation as a marker of unfavorable outcome (Kim D.Y. et al., 2009; Deng et al., 2010, 2013; Ji et al., 2016). In CRC, STAT3 is one of the major oncogenic proteins associated with proliferation, angiogenesis, invasion and chemo-radiotherapy resistance (Lin et al., 2005, 2011; Munro et al., 2018). Also, its inhibition prevents tumor initiation, being an attractive therapeutic target for CRC (Lin et al., 2011).

SALL4 (Spalt Like Transcription Factor 4)

As a TF, SALL4 plays essential roles in maintaining pluripotency and self-renewal of ESCs, being downregulated or silenced in differentiated cells (Zhang et al., 2006, 2015; Yang et al., 2008). SALL4 acts as an oncogene and it is associated with cancer initiation, development, and progression (Ma et al., 2006). Zhang et al. (2014) showed that the overexpression of SALL4 is associated with gastric CSC features and could be involved in the generation and maintenance of these cells. Later, Yuan et al. (2016) suggested a novel mechanism for SALL4 role in GC, showing that this TF binds to the promoter region of CD44 and activates its expression, enhancing cell proliferation, migration and invasion. Increasing evidence indicates that upregulation of SALL4 is associated with lymph node metastasis and poorer overall prognosis (Zhang et al., 2014, 2018b). In CRC, SALL4 overexpression is detected in 87% of tumor tissues and it is correlated with tumor cell metastasis to lymph nodes being associated with poor prognosis and showing its essential role in maintaining the properties of CSCs (Forghanifard et al., 2013; Zhang et al., 2015).

β -Catenin (Catenin Beta 1)

The Wnt/ β -catenin pathway is implicated in the regulation of the epithelial stem cell self-renewal (Behrens et al., 1996). Alone, β -catenin signaling has been shown as necessary for the maintenance of CSC features (Huang et al., 2007; Kanwar et al., 2010; Jiang R. et al., 2016). The dysregulation of the Wnt/ β -catenin signaling pathway has been implicated in colon carcinogenesis and plays a critical role in regulating the growth and maintenance of colonospheres (Kolligs et al., 2002; Kanwar et al., 2010). The activation of this pathway can lead to the

conversion of intestinal stem cells into CSCs, where expression levels of β -catenin are higher (Kanwar et al., 2010). Some studies revealed that high levels of nuclear β -catenin, in CRC patients, were associated with a poor prognosis and could be used as a biomarker for late phase CRC (Chen Z. et al., 2013). Gastric CSCs self-renewal and proliferation ability, both *in vitro* and *in vivo*, are also improved by the Wnt/ β -catenin signaling (Mao et al., 2014; Chiurillo, 2015).

CSCs AND TUMOR MICROENVIRONMENT

The close interaction between CSCs and their niche is fundamental for maintaining the stemness of CSCs and tumor progression. The CSC niche, a specific tumor microenvironment, which consists of stroma, micro-vessels, hypoxic regions, cancer-associated fibroblasts (CAFs), cancer-associated mesenchymal stem cells (MSCs), tumor-associated macrophages (TAMs) and extracellular matrix, secretes soluble factors (e.g., cytokines and growth factors) that are necessary for cancer cell survival (Quante et al., 2013; Lau et al., 2017; Yang et al., 2020). Growth factors and cytokines regulate Wnt, Notch, JAK-STAT3 and other signaling pathways thereby stimulating growth, epithelial-to-mesenchymal transition (EMT), invasion, angiogenesis, metastasis and inhibiting apoptosis (Yang et al., 2020). These pathways are required for the self-renewal and maintenance of CSCs. For instance, growth factors like hepatocyte growth factor (HGF), secreted by the stromal myofibroblasts, activate the Wnt-signaling in a subset of colon cancer cells that maintain the CSC phenotype (Vermeulen et al., 2010). Another example is hypoxia, which also maintains a stem-like phenotype in CRC and GC through the increased expression of hypoxia-inducible factors (HIFs), the transcription factors HIF-1 α and HIF-2 α , that maintain the Wnt/ β -catenin signaling pathway and activate stemness-related TFs such as OCT4 (Gidekel et al., 2003; Liu et al., 2008; Mazumdar et al., 2010; Yeung et al., 2011; Vadde et al., 2017). On the other hand, cancer cells also secrete growth factors and proteases to promote changes in their microenvironment (Ishimoto et al., 2014). One significant example of this crosstalk between cancer cells and the microenvironment is the secretion of cytokines like interleukin-6 (IL-6) by the cancer-associated mesenchymal stem cells that enhance the progression of CRC through the IL-6/JAK2/STAT3 signaling (Zhang et al., 2018a). The same is observed in GC through the secretion of interleukin-8 (Li W. et al., 2015). On the other hand, cancer cells mediate the production of inflammatory cytokines with pro-tumorigenic roles or the inhibition of cytokines involved in immune surveillance, altering the composition of the immune cells in the tumor microenvironment (Quante et al., 2013; West et al., 2015). Specifically, Rezaei et al. (2019) suggested that CSCs could alter the cytokines in the tumor microenvironment by demonstrating that the balance between suppressive regulatory T cells (Treg) and T helper cells producing IL17 (Th17) could be affected. Chaudhry et al. (2009) disclosed further that STAT3 is fundamental for the inhibition of Treg cells development and

Th17 differentiation. In fact, the STAT3 transcription factor, in collaboration with NF κ B, regulates the expression of these genes encoding critical cancer-promoting inflammatory mediators, establishing a crosstalk between cancer and immune cells of the microenvironment and perpetuating the effects of STAT3 activation in cancer cells (Yu et al., 2007; Grivennikov and Karin, 2010; Yang et al., 2019). However, despite the growing evidences on the interaction of gastric and colorectal CSCs with the tumor microenvironment, the specific molecules involved and their signaling pathways still need further investigation in order to design safe therapies.

THERAPEUTIC APPROACHES TO TARGET CSCs

The therapeutic approach in GC and CRC is determined by the stage of the disease at the time of diagnosis. Patients are treated with surgery, chemotherapy and/or radiation. In GC, surgery remains the main treatment for stage I. It can also be performed at stages II and III but chemotherapy (perioperative, neoadjuvant or adjuvant) is necessary to improve overall survival of the patients. For stage IV, chemotherapy with doublet or triplet platinum/fluoro-pyrimidine combinations or capecitabine is the main treatment (Neri et al., 2007; Smyth et al., 2016). Resective surgery is the main curative treatment used in non-metastasized CRC, although neo-adjuvant treatments are also administered in rectal carcinoma (Kuipers et al., 2015). After surgery, 5-FU-based chemotherapy is used to reduce the risk of tumor recurrence and improve overall survival of patients (Dienstmann et al., 2017). Currently, the decision of giving adjuvant treatment to early-stage CRC patients is recommended to high risk patients with one or more risk factors (Kannarkatt et al., 2017). Patients with very high risk – microsatellite stable (MSS) and T4 may be considered for the addition of oxaliplatin (Labianca et al., 2013). However, in some cases of GC and CRC, after a believed efficacious treatment, the cancer reappears locally or in distant metastasis. This results from the presence of CSCs that were able to resist the therapy applied, revealing that the existence of CSCs is one of the biggest difficulties in cancer treatment (Dean et al., 2005). Thus, direct targeting of CSCs seems to be the key for tumor complete elimination. The therapy against CSCs using specific molecules should eradicate these cells while the conventional therapy eliminates the non-CSCs present in the tumor bulk. Nevertheless, this type of treatment should be administrated in combination due to the possibility of cell plasticity that facilitates the appearance of *de novo* CSCs from non-CSCs. Moreover, targeting specific TFs or signaling pathways responsible for maintaining the CSC phenotype could become novel therapies against GC and CRC. For that reason, several clinical trials are being undertaken to explore the efficacy of diverse compounds, that are capable of modulating or inactivating proteins that gastric and colorectal CSCs use to grow and survive, allowing their elimination. Additionally, it is worth to mention that several studies have been conducted to evaluate the efficacy of small molecules in targeting CSCs *in vitro* and *in vivo*, compounds capable of eliminating or reducing the CSC population that could

also be part of the path in cancer therapy targeting CSCs (Gupta et al., 2009; Abetov et al., 2015; Shapiro et al., 2016; Müller et al., 2017; Park et al., 2017; Pádua et al., 2020). **Table 2** lists the drugs that have been or are being investigated in clinical trials, alone or in combination with other compounds, to treat GC and CRC.

Immunotherapy

Cancer immunotherapy has emerged as a potential tool for cancer treatment (Farkona et al., 2016). Several immunotherapeutic strategies have been developed including cancer vaccines, oncolytic viruses, monoclonal antibodies or recombinant proteins, chimeric antigen receptor T cell (CAR-T) cells and other cellular therapies, lymphocyte-activating cytokines and checkpoint inhibitors (Riley et al., 2019). Immunotherapy aims to improve the immune system response against cancer cells through natural mechanisms (Riley et al., 2019). Therefore, it can be used to target cancer cells and also CSCs in the tumor microenvironment (Badrinath and Yoo, 2019). Many immunotherapeutic agents targeting CSCs have been tested in clinical trials (Menon et al., 2016). Monoclonal antibodies that specifically target CSC surface biomarkers have been used in gastric and colorectal cancer. Ongoing there is a Phase I study of RO5429083, a monoclonal antibody against CD44 in patients with metastatic and/or locally advanced malignant solid tumors (NCT01358903). Also used were EpCAM antibodies, such as adrecolomab, that was tested in patients with resected stage II adenocarcinoma of the colon (Niedzwiecki et al., 2011) and adecatumumab, that is being tested in a Phase II study to evaluate efficacy and safety, alone or with FOLFOX, in metastasized CRC (NCT00866944). A Phase I dose finding study is evaluating the bispecific antibody targeting EGFR and Lgr5 (MCLA-158) in metastatic CRC and other advanced solid tumors (NCT03526835). In addition, CAR-T cell therapies, have been developed to target CSCs in GC and CRC. There are three Phase I or II clinical trials in GC using CAR-T cells targeting EpCAM (NCT03013712, NCT03563326, and NCT02725125), one of them consisting in the intraperitoneal infusion in advanced GC with peritoneal metastasis. NCT03013712 includes patients with colon cancer. Moreover, there is a Phase I/II clinical study of CAR-T cells targeting CD133 in relapsed and/or chemotherapy refractory malignancies including CRC (NCT02541370).

Targeting the Transcription Factor STAT3

From the list of TFs strongly associated with CSC phenotype in GC and CRC, STAT3 became crucial as a molecular target for cancer therapy because napabucasin (BBI608), the first-in-class cancer stemness (CSCs) inhibitor that works by targeting STAT3, effectively blocks cancer relapse and metastasis in xenografted human cancers (Li Y. et al., 2015; Zhang Y. et al., 2016). Napabucasin is a naturally occurring drug orally administered. It inhibits CSC self-renewal and induces apoptosis in CSCs by targeting CSC signaling pathways (STAT3, NANOG and β -catenin) in GC (Bekaii-Saab and El-Rayes, 2017). In CRC, napabucasin has been investigated in several studies. A phase I/II clinical trial investigated napabucasin with other standard therapeutic treatments for advanced gastrointestinal malignancies (Bendell et al., 2017). Another

TABLE 2 | Complete and ongoing clinical trials of therapeutic agents targeting gastric and/or colorectal CSCs, correlated signaling pathways and the transcription factor STAT3.

Drug/Antibody	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
Immunotherapy								
RO5429083	CD44	Metastatic and/or locally advanced malignant solid tumors	NCT01358903	I	Intravenous (IV)	Completed, no results posted	November 2016	Hoffmann-La Roche
Adecatumumab alone or in combination with FOLFOT	EpCAM	R0 resection of colorectal liver metastases	NCT00866944	II	Intravenous (IV)	Completed, no results posted	November 2011	Amgen Research (Munich) GmbH
MCLA-158	EGFR and Lgr5	Advanced/metastatic solid tumors, including CRC	NCT03526835	I	Intravenous (IV)	Recruiting	August 2018	Merus N.V.
EpCAM CAR-T	EpCAM	Relapsed or refractory EpCAM positive cancer	NCT03013712	III	Intravenous (IV)	Recruiting	January 2017	First Affiliated Hospital of Chengdu Medical College
EpCAM CAR-T	EpCAM	Advanced GC with peritoneal metastasis	NCT03563326	I	Intravenous (IV)	Recruiting	September 2018	Jian-Kun Hu
EpCAM CAR-T	EpCAM	GC	NCT02725125	—	Intravenous (IV)	Unknown	March 2017	Shinobiway Cell Therapy Co., Ltd.
CART-133	CD133	Chemotherapy refractory advanced malignancies, including CRC	NCT02541370	III	Intravenous (IV)	Completed, no results posted	December 2019	Chinese PLA General Hospital
Transcription Factor inhibitors								
BB1608 or BNC105 in combination with nivolumab	STAT3	Metastatic CRC	NCT03647839	II	Oral and Intravenous (IV)	Recruiting	February 2019	Australasian Gastro-Intestinal Trials Group
Danvatirsen (AZD9150) in combination with Durvalumab (MEDI4736)	STAT3	Advanced and refractory pancreatic, non-small cell lung cancer, and mismatch repair deficient CRC	NCT02988378	II	Intravenous (IV)	Recruiting	March 2020	M.D. Anderson Cancer Center
Napabucasin (GB201) in combination with FOLFIRI	STAT3	Metastatic CRC	NCT03522649	III	Oral and Intravenous (IV)	Recruiting	June 2019	1Globe Health Institute LLC
TTI-101	STAT3	Advanced cancers, including gastric adenocarcinoma and CRC	NCT03195699	I	Oral	Recruiting	February 2020	Tvardi Therapeutics, Incorporated

(Continued)

TABLE 2 | Continued

Drug	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
Wnt/β-catenin pathway inhibitors								
Artesunate , prior to surgery	β -catenin	Stage II/III CRC	NCT02633098	II	Oral	Recruiting	April 2019	St George's, University of London
DKN-01 in combination with atezolizumab	DKK1	GC	NCT04166721	I/II	Intravenous (IV)	Recruiting	February 2020	Royal Marsden NHS Foundation Trust
ETC-1922159 in combination with pembrolizumab	PORCN	Advanced solid tumors	NCT02521844	I	Oral and Intravenous (IV)	Active, not recruiting	October 2019	EDDC (Experimental Drug Development Centre), A*STAR Research Entities
Foxy-5	Wnt-5A	CRC	NCT02020291	I	Intravenous (IV)	Completed, no results posted	February 2016	WntResearch AB
Foxy-5	Wnt-5A	Metastatic breast, colon or prostate cancer	NCT02655952	I	Intravenous (IV)	Completed, no results posted	December 2018	WntResearch AB
Foxy-5 as neo-adjuvant therapy	Wnt-5A	Colon cancer	NCT03883802	II	Intravenous (IV)	Recruiting	April 2019	WntResearch AB
Genistein in combination with FOLFOX or FOLFOX-Avastin	tyrosine kinase and topoisomerase-II	Metastatic CRC	NCT01985763	I/II	Oral and Intravenous (IV)	Completed, has results	May 2019	Sofya Pintova
Lgk974	PORCN	Malignancies dependent on Wnt ligands, including BRAF mutant CRC	NCT01351103	I	Oral	Recruiting	February 2020	Novartis Pharmaceuticals
Lgk974 in combination with LGX818 and cetuximab	PORCN	BRAF-mutant metastatic CRC and Wnt pathway mutations metastatic CRC	NCT02278133	I/II	Oral	Completed, no results posted	October 2017	Array BioPharma
MCLA-158	EGFR and LGR5	Advanced/metastatic solid tumors, including CRC	NCT03528835	I	Intravenous (IV)	Recruiting	August 2018	Merus N.V.
Mesalazine	induces the expression of μ -protocadherin	CRC	NCT02077777	II	Oral	Completed, no results posted	December 2016	SOFAR S.p.A.
OMP-18R5 (Vantictumab)	Wnt receptor	Solid tumors	NCT01608867	I	Intravenous (IV)	Completed, no results posted	September 2016	OncMed Pharmaceuticals, Inc.
OMP-54F28 (Ipatricept)	Wnt receptor	Solid tumors	NCT01608867	I	Intravenous (IV)	Completed, no results posted	July 2017	OncMed Pharmaceuticals, Inc.

(Continued)

TABLE 2 | Continued

Drug	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
Hedgehog (Hh) pathway inhibitors								
BMS-833923 (XL139)	Smoothed	Advanced or metastatic solid tumors	NCT01413906	I	Oral	Completed, no results posted	June 2013	Bristol-Myers Squibb
BMS-833923 in combination with cisplatin and capecitabine	Smoothed	Metastatic gastric, gastroesophageal, or esophageal adenocarcinomas	NCT00909402	I	Oral and Intravenous (IV)	Completed, no results posted	June 2013	Bristol-Myers Squibb
LDE255 (Sonidegib) in combination with BKM120 (Buparlisib)	Smoothed (and PI3K)	Advanced solid tumors	NCT01576666	I	Oral	Completed, no results posted	February 2016	Novartis Pharmaceuticals
LEQ-506	Smoothed	Advanced solid tumors	NCT01106508	I	Oral	Completed, no results posted	February 2020	Novartis Pharmaceuticals
Vismodegib in combination with standard chemotherapy	Smoothed	Advanced GC or gastroesophageal junction cancer	NCT00982592	II	Oral and Intravenous (IV)	Completed, has results	January 2016	National Cancer Institute (NCI)
Vismodegib (GDC-0449) with concurrent chemotherapy and bevacizumab as first-line therapy	Smoothed	Metastatic CRC	NCT00636610	II	Oral	Completed, has results	June 2017	Genentech, Inc.
NOTCH pathway inhibitors								
BMS-906024	Pan-Notch	Advanced or metastatic solid tumors	NCT01292655	I	Intravenous (IV)	Completed, no results posted	January 2020	Bristol-Myers Squibb
CB-103	Pan-Notch	Advanced or metastatic solid tumors, including CRC	NCT03422679	I/II	Oral	Recruiting	May 2019	Celastia Biotech AG
LY3039478 (Creigacestat)	Pan-Notch	Advanced solid tumors	NCT02836600	I	Oral	Active, not recruiting	December 2019	Eli Lilly and Company
LY3039478 (Creigacestat) in combination with other anticancer agents	Pan-Notch	Advanced or metastatic solid tumors	NCT02784795	I	Oral and Intravenous (IV)	Completed, no results posted	March 2020	Eli Lilly and Company
LY900009	γ -Secretase	Advanced cancer	NCT01158404	I	Oral	Completed, has results	August 2019	Eli Lilly and Company
MED10639	DLL4	Advanced solid tumors	NCT01577745	I	Intravenous (IV)	Completed, has results	May 2017	MedImmune LLC
REGN421 (SAR153192; Enoticumab)	DLL4	Advanced solid malignancies	NCT00871559	I	Intravenous (IV)	Completed, no results posted	March 2014	Regeneron Pharmaceuticals

(Continued)

TABLE 2 | Continued

Drug	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
R04929097	γ -Secretase	Recurrent and metastatic CRC	NCT01116887	II	Oral	Completed, has results	May 2014	National Cancer Institute (NCI)
R04929097 in combination with capecitabine	γ -Secretase	Refractory solid tumors	NCT01158274	I	Oral	Completed, no results posted	November 2014	National Cancer Institute (NCI)
R04929097 in combination with cediranib maleate	γ -Secretase	Advanced solid tumors including CRC	NCT01131234	I	Oral	Completed, no results posted	December 2014	National Cancer Institute (NCI)
PI3K/AKT/mTOR pathway inhibitors								
Acetylsalicylic acid (aspirin)	—	CRC stage I–III with mutations in the PI3K signaling pathway	NCT02647099	III	Oral	Recruiting	August 2019	Anna Marling
Acetylsalicylic acid (aspirin)	—	Resected colon cancer with PI3K mutation stage II or III high risk	NCT02945033	III	Oral	Recruiting	October 2019	University Hospital, Rouen
Acetylsalicylic acid (aspirin)	—	Dukes C and high-risk dukes B CRCs	NCT005665708	III	Oral	Recruiting	September 2019	National Cancer Centre, Singapore
Acetylsalicylic acid (aspirin) and metformin	—	Stage I–III CRC patients	NCT03047837	II	Oral	Recruiting	February 2019	Erte Ospedallero Ospedali Galliera
AZD2014 (Vistusertib)	mTORC1 and mTORC2	PI3K amplified GC	NCT03061708	II	Oral	Terminated (lack of efficacy)	May 2019	Samsung Medical Center
AZD2014 (Vistusertib)	mTORC1 and mTORC2	TSC1/2 mutated or TSC1/2 null GC	NCT03082833	II	Oral	Terminated (lack of efficacy)	May 2019	Samsung Medical Center
AZD5363 in combination with paclitaxel	AKT	Advanced gastric adenocarcinoma	NCT02451956	II	Oral and Intravenous (IV)	Completed, no results posted	December 2019	Samsung Medical Center
AZD8186 in combination with paclitaxel	PI3K β/δ	Advanced GC	NCT04001569	I/II	Oral and Intravenous (IV)	Recruiting	June 2019	Seoul National University Bundang Hospital
BKM120 (Buparlisib) in combination with LDE255 (Sonidegib)	PI3K and Smoothed	Advanced solid tumors	NCT01576666	I	Oral	Completed, no results posted	February 2016	Novartis Pharmaceuticals
BYL719 (Alpelisib) in combination with AU922	PI3K	Advanced or metastatic GC	NCT01613950	I	Oral and Intravenous (IV)	Completed, no results posted	February 2020	Novartis Pharmaceuticals
BYL719 (Alpelisib) in combination with LGX818 (Ercorafenib) and Cetuximab	BRAF, EGFR and PI3K	in BRAF Mutant Metastatic CRC	NCT01719380	I/II	Oral and Intravenous (IV)	Completed, no results posted	April 2019	Array BioPharma

(Continued)

TABLE 2 | Continued

Drug	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
Cabozantinib	Multikinases	Refractory metastatic CRC	NCT03542877	II	Oral	Active, not recruiting	April 2019	Academic Thoracic Oncology Medical Investigators Consortium
CB-839 (Telaglenastat) in combination with capecitabine	Glutaminase	Solid tumors and fluoropyrimidine resistant PIK3CA mutant CRC	NCT02861300	I/II	Oral	Recruiting	March 2020	Case Comprehensive Cancer Center
Copanlisib in combination with anti-PD-1 antibody Nivolumab	PI3K	Relapsed/refractory solid tumors, mismatch-repair proficient (MSS) CRC	NCT03711058	I/II	Intravenous (IV)	Recruiting	July 2019	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
DS-7423	PI3K and mTOR	Advanced solid malignant tumors	NCT01364844	I	Oral	Completed, no results posted	February 2014	Daiichi Sankyo, Inc.
Ipatasertib (GDC-0068) in combination with oxaliplatin, 5-FU and leucovorin	AKT	Advanced or metastatic GC or gastroesophageal junction cancer	NCT01896531	II	Oral and intravenous (IV)	Active, not recruiting	February 2020	Genentech, Inc.
MK-2206	AKT	Advanced GC or gastroesophageal junction cancer	NCT01260701	II	Oral	Completed, has results	January 2016	National Cancer Institute (NCI)
MK-2206	AKT	CRC that is metastatic or locally advanced and cannot be removed by surgery	NCT01802320	II	Oral	Completed, has results	September 2019	National Cancer Institute (NCI)
MK-2206 in combination with AZD6244 (Selumetinib)	AKT and MEK	Advanced CRC	NCT01333475	II	Oral	Completed, has results	September 2015	National Cancer Institute (NCI)
Neratinib in combination with Trastuzumab or with Cetuximab	HER2 and EGFR	KRAS/NRAS/BRAF/PIK3CA wild-type metastatic CRC by HER2 status	NCT03457896	II	Oral	Recruiting	November 2019	NSABP Foundation Inc
Pembrolizumab (MK-3475) combined with INCB050465 or with Itactinib (INCB039110)	JAK1 and PI3K-delta	Advanced solid tumors, including CRC	NCT02646748	I	Oral	Active, not recruiting	December 2019	Incyte Corporation

(Continued)

TABLE 2 | Continued

Drug	Targets	Disease/Condition	NCT Identifier	Phase	Routes of administration	Recruitment status	Last update	Sponsor
PX-866 (Sonolisib) in combination with Cetuximab	PI3K	Incurable metastatic CRC	NCT01252628	I/II	Oral	Completed	May 2018	Cascade Therapeutics Inc.
RAD001 (Everolimus)	TOR serine-threonine kinases	Previously treated unresectable or metastatic esophageal cancer or GC	NCT00985192	II	Oral	Completed, has results	February 2020	Translational Oncology Research International
RAD001 (Everolimus)	TOR serine-threonine kinases	Advanced GC	NCT00519324	II	Oral	Completed, no results posted	November 2015	Novartis Pharmaceuticals
RAD001 (Everolimus) in combination with AV-951 (Tivozanib)	TOR serine-threonine kinases and VEGFRs 1, 2, and 3	Gastrointestinal cancer	NCT01058655	I/II	Oral	Completed, has results	April 2017	Dana-Farber Cancer Institute
RAD001 (Everolimus) in combination with Capecitabine and Oxaliplatin	TOR serine-threonine kinases	Advanced GC	NCT01049620	I	Oral and Intravenous (IV)	Completed, no results posted	January 2020	Asan Medical Center
RAD001 (Everolimus) in combination with displatein and HDLF (high-dose 5-FU and leucovorin)	TOR serine-threonine kinases	Advanced GC	NCT00632268	II	Oral and Intravenous (IV)	Completed, no results posted	August 2013	National Taiwan University Hospital
SAR245409 (Vortalisib) in combination with MSC1936369B (gimasertib)	PI3K, MTOR, MEK1 and 2	Advanced or metastatic solid tumors (GC not included)	NCT01390818	I	Oral	Completed, has results	March 2017	EMD Serono
Serabelisib in combination with Canagliflozin	PI3K α and SGLT2	Advanced solid tumors	NCT04073680	I/II	Oral	Not yet recruiting	February 2020	Petra Pharma
SF-1126	PI3K	Solid tumors	NCT00907205	I	Intravenous (IV)	Completed, no results posted	June 2013	Semafore Pharmaceuticals
Trametinib in combination with Trifluridine and Tipiracil Hydrochloride	MEK	Chemotherapy-resistant RAS-mutated (PIK3CA/PTEN-Wild-Type) metastatic CRC	NCT03317119	I	Oral	Recruiting	January 2020	City of Hope Medical Center

GC corresponds to gastric cancer and CRC to colorectal cancer. FOLFIRI corresponds to a chemotherapy regimen with folinic acid (leucovorin), fluorouracil (5-FU) and oxaliplatin (Eloxatin) and FOLFIRI corresponds to a chemotherapy regimen with folinic acid (leucovorin), fluorouracil (5-FU) and irinotecan (Camptosar).

study – CanStem303C – a randomized phase III clinical trial done in adult patients with previously treated metastatic CRC evaluated napabucasin in combination with FOLFIRI (Grothey et al., 2017). Napabucasin monotherapy has been reported in a published phase III trial. The CO.23 trial evaluated the efficacy of napabucasin monotherapy versus placebo in metastasized CRC, which failed to demonstrate a significant difference in the napabucasin group survival. However, in a pre-specified biomarker analysis, phosphorylated STAT3 (pSTAT3)-positive patients experienced a significant survival benefit from napabucasin over placebo (Jonker et al., 2018; Sonbol et al., 2019). Another ongoing trial involving STAT3 inhibition is the phase II trial MODULATE (NCT03647839) which specifically aims to study the modulation of the tumor microenvironment using either vascular disrupting agents (BNC105) or STAT3 inhibition (BB1608), in synergy with an immune checkpoint protein (PD1) inhibitor (nivolumab). This trial is recruiting microsatellite stable, refractory CRC cases.

Targeting the Wnt/ β -Catenin Signaling Pathway

Wnt/ β -catenin signaling pathway is a major regulator of normal intestinal development and its over-activation behaves as a hallmark of CRC, being particularly significant in drug resistance and stemness maintenance of colorectal CSCs (Takebe et al., 2011; Basu et al., 2016). The activation of the Wnt/ β -catenin signaling pathway is associated with poor prognosis (Janssen et al., 2006). It results mostly from the accumulation of mutations in the APC tumor suppressor gene, oncogenic KRAS-signaling pathway, β -catenin and p53. Mutations that do not allow the formation of the APC/Axin/GSK3 β destruction complex result in the accumulation and nuclear translocation of β -catenin that binds to transcription factors of T cell factor family (TCF4) and activates target genes like c-Myc, cyclinD1 and survivin, some involved in maintaining stemness (Myant et al., 2013; Lee et al., 2015; Zhou et al., 2017). It has been shown that loss of APC in CRC triggered the expression of a Rac1 GTPase via the induced expression of c-Myc, necessary to intestinal stem cell proliferation and CRC initiation (Myant et al., 2013). On the other hand, p53 may affect the outcome of Wnt signaling in CRC development (Voorneveld et al., 2015), having a role in the acquisition of pluripotency during reprogramming (Takahashi and Yamanaka, 2006; Krizhanovsky and Lowe, 2009). The Wnt signaling pathway is also dysregulated in GC (Chiurillo, 2015). However, the involvement and mechanisms are not yet as fully understood as in CRC. A number of studies suggest β -catenin and APC as driver genes, revealing somatic mutations in both genes that might have relevance in GC (Hori et al., 1992; Nakatsuru et al., 1993; Woo et al., 2001; Clements et al., 2002; Zhang and Xue, 2008). Genomic analysis of several gastric primary tumors disclosed that Wnt/ β -catenin, together with NF- κ B and proliferation/stem cell pathways, were deregulated in more than 70% of the primary tumors. Patient stratification by combinations of these oncogenic pathways revealed to be a great tool for GC clinical behavior assessment (Ooi et al., 2009). Many molecules have been used to target the Wnt/ β -catenin in gastrointestinal

CSCs (Chiurillo, 2015; Parizadeh et al., 2019; Patel et al., 2019; Yang et al., 2020). Genistein, a soy-derived compound, was tested in a Phase I/II research trial, in combination with FOLFOX or FOLFOX-Bevacizumab, where it was demonstrated to be well tolerated by patients and may have improved efficacy in the treatment of metastatic CRC (Pintova et al., 2019). *In vitro* and *in vivo* studies have shown that Genistein affects mainly Wnt/ β -catenin and PI3K/AKT pathways (Su et al., 2003; Kim et al., 2005; Zhang and Chen, 2011; Wang et al., 2012).

Targeting the Hedgehog Signaling Pathway

The dysregulation of Hedgehog (Hh) signaling pathway has been reported as another main cause of CSCs self-renewal and chemoresistance, being associated with poor clinical outcome in patients with GC or CRC (Ma et al., 2005; Varnat et al., 2009; Takebe et al., 2011; Usui et al., 2018). In this pathway, target gene expression is predominantly regulated by the Smoothened (SMO) protein but GLI inhibitors are also used (Rimkus et al., 2016; Didiasova et al., 2018). In GC cells, SMO regulates nuclear translocation of GLI-1 that in turn promotes transcription of target genes, such as CD44 (Yoon et al., 2014). Yoon et al. (2014) showed that in AGS, MKN-45, and NCI-N87 GC cell lines, the Hh pathway inhibition using SMO shRNA or small-molecule inhibitors significantly decreased spheroid formation ability and tumor growth. Vismodegib (GDC-0449), the first Hh pathway inhibitor used in cancer research, is currently undergoing Phase II trials in advanced GC and in metastatic CRC (Gupta et al., 2010; Berlin et al., 2013). Examination of tumor samples revealed that Vismodegib has not increased progression-free or overall survival as a whole, but only in a limited subset of patients with high CD44 expression (Cohen et al., 2013; Yoon et al., 2014). Disappointingly, these treatments with Vismodegib, did not increase progression-free survival in CRC patients (Low and de Sauvage, 2010; McMillan and Matsui, 2012). When the SMO inhibitor AY9944 was used in combination with the GLI-1 inhibitor GANT61, there was an increased response to anti-cancer drugs in tumor organoids and a decreased capacity to form colonies in SW480 and HCT116 CRC cells (Usui et al., 2018). These results indicate that this strategy might be worthwhile in CRC.

Targeting the Notch Signaling Pathway

The Notch signaling pathway is one of the most activated signaling pathways in cancer, namely in GC and CRC, and promotes metastization (Du et al., 2014; Hayakawa et al., 2019). It has a key role in the maintenance and differentiation of CSCs (Quail et al., 2012; Lu et al., 2013; Yang et al., 2020). In GC and CRC, the expression of Notch1 or Jagged1 is associated with poor prognosis (Yeh et al., 2009; Kang et al., 2012; Jackstadt et al., 2019; Kim et al., 2019; Mohamed et al., 2019). In CRC, Notch1 is associated with more aggressive subtypes, recruiting neutrophils to drive metastasis (Jackstadt et al., 2019). Due to the fact that the Notch pathway is related to CSC self-renewal and angiogenesis, targeting this pathway became a potential anti-CSC therapeutic approach (Venkatesh et al., 2018). Strategies

used to inhibit Notch signaling include γ -secretase inhibition (GSI), Notch receptor (e.g., Notch1, Notch2, and Notch3) or ligand (e.g., Jagged1 and Jagged2) antibodies and combination therapy with inhibitors of other pathways. The inhibition of Notch receptors using two GSIs has allowed to disclose the importance of Notch pathway in the growth and survival of GC cells (Brzozowa et al., 2013). Furthermore, GSIs lead to the induction of apoptosis and inhibition of tumor-sphere formation of CD44⁺ gastric CSCs (Barat et al., 2017). However, it is important to mention that GSIs do not only target Notch-related proteins but also proteases involved in numerous cellular processes, which could originate adverse effects *in vivo* (Shih et al., 2007; Wang et al., 2010; Brzozowa et al., 2013). Nevertheless, various clinical trials have been performed to evaluate the efficacy of targeting the Notch signaling pathway in GC and CRC (Table 2). RO4929097, a selective GSI, showed good anti-tumor activity in preclinical and early trials but it was not good enough for metastatic CRC (Luistro et al., 2009; Strosberg et al., 2012; Tolcher et al., 2012). Combinations of RO4929097 with other drugs in advanced solid tumors, including CRC, were well tolerated and presented some clinical benefit (Sahebjam et al., 2013; LoConte et al., 2015). LY900009 is another GSI tested in advanced cancers, including those of gastrointestinal tract. It is currently in a phase I trial and revealed a safety profile, with the majority of patients experiencing low-grade gastrointestinal adverse effects (Pant et al., 2016). Furthermore, it was demonstrated to have an inhibitory effect on Notch signaling pathway, inducing goblet cell differentiation and increased mucin production, similarly to that observed in rats (Pant et al., 2016). Moreover, MEDI0639 is a monoclonal antibody that specifically binds to DLL4 and prevents its interaction with Notch receptors, thereby inhibiting Notch-mediated signaling and target gene transcription, which may block tumor angiogenesis and eventually tumor cell growth (Ishigami et al., 2013). A phase I study in advanced solid tumors demonstrated that MEDI0639 is well tolerated and preliminary results show evidence of antitumor activity (Falchook et al., 2015).

Targeting the PI3K/AKT/mTOR Signaling Pathway

The PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathway is typically abnormally activated in many carcinomas (Michl and Downward, 2005; Johnson et al., 2010; Narayanankutty, 2019). It is thought to be crucial in angiogenesis, cell proliferation, metabolism, survival, metastasis and drug resistance (Cantley, 2002; Edinger and Thompson, 2002; Fingar et al., 2004; Al-Batran et al., 2012; Tapia et al., 2014). AKT is commonly overexpressed in tumors and plays an important role in the metabolic reprogramming of cancer (Yap et al., 2011; Iida et al., 2013). Although the PI3K/AKT/mTOR pathway has been extensively studied, there are few studies in CSCs. In GC and CRC, activation of mTOR appears to cause tumor progression and poor patient survival (Lang et al., 2007; Murayama et al., 2009; Xiao et al., 2009; Yu G. et al., 2009; An et al., 2010). Thus, its inhibition seems to be fundamental for GC and CRC therapy. A phase II study using RAD001

(everolimus) in previously treated unresectable or metastatic esophageal cancer or GC was performed over the rationale that everolimus may stop tumor growth by blocking some of the fundamental enzymes for cell growth and by blocking angiogenesis. Everolimus was well tolerated by the patients however this study displayed a strong weakness by being single-arm and non-comparative. However, mTOR suppression decreased ALDH1 activity, which is a marker of CSCs in CRC (Xia and Xu, 2015). Growth inhibition, using a dual PI3K/mTOR inhibitor, PF-04691502, was observed *in vitro* and in xenografted CRC tumors (Fang et al., 2013). Another mTOR inhibitor decreased survival and invasion of colorectal CSCs *in vitro*, and suppressed tumor growth *in vivo* (Francipane and Lagasse, 2013). The allosteric AKT inhibitor (MK-2206) led to a decrease in CSCs proliferation, and reduction of the capacity to form colonospheres *in vitro* and to initiate tumor formation *in vivo*. Mice with xenografted tumors showed a significant decrease in tumor progression. Also, MK-2206 significantly inhibited the growth of patient-derived tumorspheres (Malkomes et al., 2016). A phase II study in advanced gastric or gastroesophageal junction cancer has revealed that MK-2206, as second-line therapy, at a dose of 60 mg was well tolerated by patients and showed some modest evidence of activity, however, the overall survival (5.1 months) was lower than the study efficacy endpoint (6.5 months) (Ramanathan et al., 2015). However, there is a Phase II clinical trial with MK-2206, to study patients with previously treated colon or rectal cancer that has spread and cannot be removed by surgery, concluding that in contrast to robust preclinical data, it does not have effect in these tumors (Dasari et al., 2016). MK-2206 was also used in combination with AZD6244 (Selumetinib), a mitogen-activated protein extracellular signal-regulated kinase (MEK)1/2 inhibitor, in a Phase II trial but the level of target inhibition obtained with the maximum non-toxic dose was not the expected (Do et al., 2015). SAR245409 (Voxalisib) was tested in a Phase I research trial in combination with another MEK inhibitor, MSC1936369B (Pimasertib), in advanced or metastatic solid tumors (Schram et al., 2018). The primary purpose of the study was to determine the maximum tolerated dose of the drug combination. The drug RAD001 (Everolimus) downregulates mTOR. A combination of RAD001, mFOLFOX-6 and Bevacizumab has been shown to be efficacious and safe in metastatic CRC (Weldon Gilcrease et al., 2019). A multicenter phase II study for patients with refractory, metastatic CRC concluded good tolerability and efficacy of Everolimus combined with Tivozanib (an oral VEGF receptor-1, -2, -3 inhibitor) with 50% of the patients having stable disease at 2 months (Wolpin et al., 2013).

DISCUSSION

The identification of CSCs remains a challenging task, particularly in solid tumors like GC and CRC. The use of cell surface markers as a primary tool to identify gastric and colorectal CSCs has disclosed some weaknesses and for that reason the uncovering of more reliable biomarkers must become a priority.

These biomarkers should include the TFs that are required for the maintenance of gastric and colorectal CSCs phenotype, as well as components of the signaling pathways that have key roles in CSC features. This explains why TFs such as STAT3 and signaling pathways like Wnt/ β -catenin, Hedgehog, NOTCH and PI3K/AKT/mTOR emerged as powerful targets, whose inactivation or modulation could eliminate gastric and colorectal CSCs. This fact is corroborated by several completed and ongoing clinical trials targeting these potential biomarkers in both tumor types, where some of the molecules have shown promising results. The incapacity to achieve the wanted levels of target inhibition was the major shortcoming of the clinical trials. Yet, the use of higher doses is not possible due to toxicity problems, which led to the development of combinations of drugs targeting different pathways. Furthermore, it is more advisable to measure the outcome of the treatments in terms of CSCs behavior, by assessing capacity to metastasize and re-growth after removing the drug.

FUTURE PERSPECTIVES

The validation of potential gastric and colorectal CSCs biomarkers and their association with GC and CRC stage is imperative to understand patient prognosis and apply a more suitable therapy. The development of a robust therapy combining CSC targets with conventional chemotherapy could

be the solution to overcome resistance to anti-cancer drugs and completely eliminate cancer. Considering these issues, it is crucial that future studies further explore the role of TFs and components of signaling pathways on cancer stemness in order to develop therapies that could eradicate CSCs.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the FEDER – Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 – Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through the FCT – Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274); projects POCI-01-0145-FEDER-029017 and POCI-01-0145-FEDER-016390 funded by FCT. DP acknowledges FCT for financial support through a Ph.D. fellowship (SFRH/BD/146186/2019).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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