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Unveiling the transcriptional network behind gastric cancer stem cells phenotype

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UNVEILING THE TRANSCRIPTIONAL NETWORK BEHIND GASTRIC CANCER STEM CELLS PHENOTYPE

HMGA1 AS A POTENTIAL PLAYER

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"What would life be if we had no courage to attempt anything?"

Vincent van Gogh

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Abstract

Gastric cancer (GC) remains a major health problem with high incidence and mortality rates worldwide. The presence of a small subpopulation of cancer stem cells (CSCs) within the tumor has been implicated as the main reason for disease relapse and therapy resistance, leading to poorer overall prognosis. These cells usually display deregulation and activation of core stemness signaling pathways, ultimately controlled by a complex network of transcription factors (TFs). Thus, identifying this network is vital and became a crucial goal for cancer prognosis and treatment. SOX2, OCT4, KLF4 and C-MYC are known to be key TFs promoting cell stem properties, though recently new key players start to emerge. HMGA1 is an architectural chromatin protein commonly overexpressed in GC and associated with stem cell properties including increased proliferation, migration ability and epithelial-mesenchymal transition. It has also been reported to bind and regulate key reprogramming regulators such as SOX2, OCT4, KLF4, NANOG and C-MYC. Here we show for the first time that HMGA1 is upregulated on two GC cell lines with a CSC phenotype and that its overexpression on non-CSCs can activate the expression of SOX2 and C-MYC, increasing the cells ability to proliferate. However, no significant consequence was seen in 5-FU resistance, what might suggest that for a complete reprogramming more TFs are needed. Considering this, we performed a protocol optimization for co-transducing several TFs into gastric non-CSCs cells. Altogether, our results disclose HMGA1 as a potential player acting in gastric CSC reprogramming, possibly through a regulatory HMGA1/SOX2/C-MYC axis.

Keywords: gastric cancer, cancer stem cells, transcription factors, HMGA1, SOX2, C-MYC, cellular reprogramming

Resumo

O cancro do estômago mantém-se como um grande problema de saúde a nível mundial, com elevadas taxas de incidência e mortalidade. A presença de uma pequena subpopulação de células estaminais do cancro no tumor é frequentemente implicada como a principal causa de recidiva da doença e resistência à terapia, levando a um pior prognóstico dos pacientes. Estas células apresentam frequentemente desregulação e ativação de vias de sinalização chave, reguladas por uma complexa rede de fatores de transcrição. Assim, a identificação desta rede transcricional é vital, condicionando o prognóstico e o tratamento da doença. Os genes SOX2, OCT4, KLF4 e C-MYC são reconhecidos como fatores de transcrição chave na aquisição de propriedades estaminais, embora recentemente novos intervenientes destas vias comecem a emergir. O HMGA1 é uma proteína estrutural da cromatina frequentemente sobrexpresso em cancro do estômago e associado a características de células estaminais do cancro, como o aumento da capacidade proliferativa e migratória, bem como o potencial de transição epitelial-mesenquimal. Está também descrito que este fator se liga e regula importantes reguladores do processo de reprogramação, como o SOX2, OCT4, KLF4, NANOG e C-MYC. No presente estudo, mostramos pela primeira vez que o HMGA1 está sobrexpresso em duas linhas de cancro do estômago com um fenótipo estaminal e que a sua sobrexpressão em células tumorais resulta na ativação da expressão de SOX2 e C-MYC, bem como no aumento da capacidade proliferativa das células. Contudo, não foram observadas consequências significativas na resistência ao fármaco 5-FU, o que pode sugerir que para uma reprogramação mais eficiente são necessários fatores de transcrição adicionais. Com isto em mente, procedeu-se à otimização do protocolo de co-transdução de vários fatores de transcrição em células do cancro do estômago que não apresentam um fenótipo estaminal. De um modo geral, os resultados obtidos parecem apontar o HGMA1 como um fator vital para a reprogramação de células estaminais do cancro, possivelmente através de um eixo regulatório HMGA1/SOX2/C-MYC.

Palavras-chave: cancro do estômago, células tumorais estaminais, fatores de transcrição, HMGA1, SOX2, C-MYC, reprogramação celular

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LIST OF ABBREVIATIONS

5-FU: 5-fluorouacil

ALDH1: aldehyde dehydrogenase 1

BrdU: bromodeoxyuridine

BSA: bovine serum albumin

C-MYC: MYC Proto-Oncogene, bHLH Transcription Factor

CSC(s): cancer stem cell(s)

DEPC: diethyl pyrocarbonate

DMEM: dulbecco's modified eagle's medium

dNTPs: deoxyribonucleotide triphosphate

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

EMT: epithelial-mesenchymal transition

ESCs: embryonic stem cells

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

g: relative centrifugal force

GATA6: GATA-binding factor 6

GC: gastric cancer

GFP: green fluorescent protein

h: hour(s)

HCl: hydrochloric acid

HER2: Human Epidermal growth factor Receptor 2

HMGA1: High Mobility Group AT-Hook 1

HRP: horseradish peroxidase

IC_{50}: half maximal inhibitory concentration

iPSCs: induced pluripotent stem cells

KLF4: Kruppel-like Factor 4

KLF5: Kruppel-like factor 5

min: minute(s)

Na_3VO_4: sodium orthovanadate

NaCl: sodium chloride

NF-kB: Nuclear factor-κB

OCT4: Octamer-binding Transcription Factor 4

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

Pen Strep: penicillin streptomycin

PMSF: phenylmethylsulfonyl fluoride

RELA: RELA proto-oncogene, NF-kB subunit

rpm: rotations per minute

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

sec: second(s)

SOX2: SRY-Box Transcription Factor 2

TBS: Tris-buffered saline

TCA: trichoroacetic acid

TF(s): transcription factor(s)

TNM: tumor node metastasis

WHO: world health organization

I. INTRODUCTION

I.1 Gastric Cancer

I.1.1 Prevalence and risk factors

Gastric cancer (GC) represents a leading cause of cancer-related death worldwide with over 1 million new cases diagnosed every year (1). Although the incidence of GC has been steadily decreasing, it still remains a major health problem. Associated risk factors include smoking, older age, chronic inflammation, family history, dietary patterns, alcohol consumption, obesity and physical inactivity, as well as *Helicobacter pylori* (*H. pylori*) infection (2). Recent studies also report that about 9% of GC cases are associated with the Epstein-Barr virus infection (3). GC incidence differs according to sex, with rates 2-fold higher in men than in women (1). Also, there is a significant differential geographic distribution: Asia, Central and Eastern Europe and South America are high-risk areas, whereas Africa, North America, Northern Europe and Australia/New Zealand are classified as low-risk areas (Figure 1) (1). Several migration studies report a strong environmental component responsible for this regional variation (4-6). Likewise, *H. pylori* strains show genotypic variations among the diverse geographic regions, which has been linked with the variation in GC epidemiology. It has been reported that the presence of the *H. pylori* East Asia strain strongly correlates with higher GC rates (7).

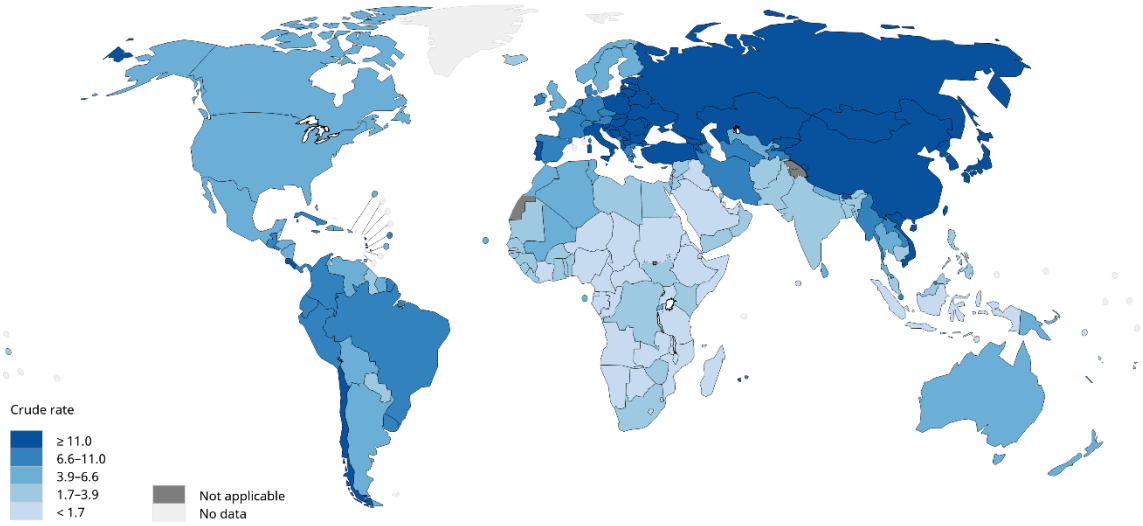

Figure 1 – Gastric cancer incidence rates. Region-specific crude incidence rates for GC in both sexes, comprising ages between 0-74. According to GLOBOCAN 2018 (1).

I.1.2 Classification

Since its establishment in 1965, the Laurén classification system has been the most widely used among all the classification systems, distinguishing two major subtypes of GC, intestinal and diffuse, which harbor microscopic and macroscopic differences (8). The intestinal type is associated with atrophic gastritis and intestinal metaplasia and is usually characterized by the presence of tubular and glandular elements, as well as cohesion between tumor cells (9). On the other hand, the diffuse type originates from normal gastric mucosa and displays single cells with little or no gland formation (9). Multiple studies show that the intestinal type is the most common, followed by the diffuse and indeterminate types (8, 10, 11).

In 2010, a new classification system arose from the World Health Organization (WHO), where gastric carcinomas are classified in four different major subtypes: tubular, papillary, mucinous and poorly cohesive, plus other uncommon histologic variants (12). The poorly cohesive carcinoma type includes the signet-ring cell carcinoma (12). In this system, the most common type of GC is the tubular adenocarcinoma, followed by papillary and mucinous types (12).

I.1.3 Diagnosis and therapeutic intervention

Early disease detection determines patients' outcomes and increases the likelihood of cure. However, GC detection is often delayed due to lack of symptoms in the early stages of the disease and because these are not disease-specific and instead often similar to minor gastrointestinal disorders, including bloating, gas, heartburn and sense of fullness (2). Patients with symptoms should undergo an upper endoscopy that, in spite of being more invasive, allows a direct histological analysis of the biopsy specimens, successfully detecting early stages of the disease (13). Pre-operative evaluations include chest and abdominal imaging to determine the feasibility of surgical resection and detect possible metastasis (13). Since many GC cases report an HER2 amplification, often associated with poorer survival, HER2 testing is recommended for all metastatic GC cases (13).

Staging of GC is done according to the TNM system based on the depth of tumor infiltration into the organ wall (T category), lymph node status (N category) and presence or absence of distant metastases (M category) (Table 1) (14).

Table 1 – Gastric cancer TNM staging system. T represents the depth of tumor infiltration into the organ wall, N is for lymph node status and M describes the presence or absence of distant metastases. Adapted from the 7th Edition of the AJCC Cancer Staging Manual (14).

Category	Definition
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa: T1a – Tumor invades lamina propria or muscularis mucosae T1b – Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures. T3 tumors also include those extending into the gastrocolic or gastrohepatic ligaments, or into the greater or lesser omentum, without perforation of the visceral peritoneum covering these structures
T4	Tumor invades serosa (visceral peritoneum) or adjacente structures: T4a – Tumor invades serosa (visceral peritoneum) T4b – Tumor invades adjacent structures such as spleen, transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine, and retroperitoneum
NX	Regional lymph node(s) cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 2 regional lymph nodes
N2	Metastasis in 3 to 6 regional lymph nodes
N3	Metastasis in 7 or more regional lymph nodes
M0	Presence of metastasis
M1	Absence of metastasis

Treatment choice for GC depends on patients' staging and time of diagnosis and so different approaches can be taken, namely endoscopic resection for superficial disease, surgical resection with lymphadenectomy, neoadjuvant/adjuvant chemotherapy, radiotherapy and palliative systemic therapy for unresectable or metastatic disease (13). More recently, new approaches have been developed, including molecular-targeted therapies - EGFR inhibitors, angiogenesis inhibitors, cell-cycle inhibitors, MMP inhibitors and HER2 inhibitors - as well as immunotherapy (15, 16).

The heterogeneity of the cancer at a molecular level plays an important role in therapy resistance and tumor recurrence, being the cancer stem cells (CSCs) among the major factors causing cancer treatment failure (13, 16).

I.2 Cancer stem cells

I.2.1 Background

Unveiling the origin of cancer has been a hot topic that might spark a light on treatment of cancer. The CSC model of tumor progression theorizes that a small subpopulation of cancer cells with stem-like properties is the core origin of tumorigenesis and sustain tumor growth, metastasis, disease relapse and therapy resistance (17). These cells are placed at the top of the hierarchy and can undergo symmetric and/or asymmetric division, having the ability to give rise to all different types of cells within the tumor (18). Although this model explains the heterogeneity of cancers concerning the hierarchical structure and progression, the origin of CSCs is still an unclear and debated topic (19, 20). Various hypotheses suggest that accordingly to the tumor type, CSCs might originate either from adult stem cells, adult progenitor cells that underwent mutation, or differentiated cells that gained stem properties through dedifferentiation (21, 22). Additionally, CSCs phenotype and functions remain arguable, with increasing evidence showing that CSCs undergo many changes as an adaptive response to the constant variation in tumor dynamics during cancer progression (23).

Early researches on CSCs focused on validating the existence of CSCs in certain tumors and finding molecular markers for their isolation. Experimental evidence of the existence of CSCs was first provided in a leukemia model in 1994, when Lapidot *et al*. identified a rare population of CD34+ CD38− leukemia-initiating cells with stem-like features that were able to transmit the disease when transplanted into severe combined immune-deficient (SCID) mice (24). Like in hematological malignancies, CSCs have also been identified in solid tumors. In 2003, Al-Hajj *et al*. isolated a CSC population in breast cancer using cell surface markers, where they disclosed that CD44+ CD24low/- ESA+ (epithelial specific antigen) cells were able to grow as a differentiated mammary carcinoma in SCID mice (25). Since then, CSCs have been found in several tumors, including brain, head and neck, melanoma, lung, prostate, pancreas, colon, ovarian, hepatic and gastric carcinoma (26, 27).

I.2.1 Gastric cancer stem cells

Evidences for gastric CSCs were initially described in 2009 when Takaishi and collaborators analyzed a panel of six GC cell lines and isolated a subpopulation with stem-

like characteristics using the cell-surface marker CD44 (28). CD44+ subpopulations of three cancer cell lines – MKN-45, MKN-74, and NCI-N87 – exhibited properties of self-renewal, spheroid colony formation, tumorigenic ability, increased therapy resistance and the capacity to form differentiated progeny giving rise to CD44- cells (28). In the other three cell lines – AGS, Kato III and MKN28 – the CD44 protein failed to mark these stem-like cells (28). Following this, several other markers have been found to be useful to identify and isolate subpopulations of gastric CSCs (Table 2) (29).

Table 2 – List of cell markers used to identify and isolate gastric CSCs.

Gastric CSCs markers	References
CD44	(28, 30)
CD24	(31)
CD54	(32-34)
EpCAM	(34-36)
CD49f	(37)
CD71	(38)
CD90	(30, 39)
CD133	(40-46)
Lgr5	(33, 47, 48)
CXCR4	(49)
ALDH1	(42, 50)

Isolation of CSCs is typically performed by fluorescence-activated cell sorting (FACS) using cell-surface markers, however, new and more robust approaches such as fluorescence reporter systems have been developed, allowing direct observation and isolation of CSCs (29). Tang and collaborators (2015) established a flexible lentiviral-based reporter system (SORE6-GFP) that responds to expression levels of the stem cell transcription factors (TFs) SOX2 and OCT4 (51). The reporter system contains six tandem repeats of a composite OCT4/SOX2 response element coupled to a minimal cytomegalovirus (CMV) promoter, used to drive expression of a fluorescent protein reporter (GFP) (51). Using this reporter system, Pádua *et al*. (2020) successfully isolated gastric CSCs from two human GC cell lines, AGS and Kato III (52). Based on the same principle, similar systems have been emerging, such as the one using a lentiviral construct carrying the promoter of NANOG to identify prostate CSCs and more recently, another using the single expression of OCT4 for isolation of breast CSCs (53, 54).

Together, these findings demonstrate that CSCs show deregulation of several molecules involved in core stemness signaling pathways, that can be used to isolate and characterize them. The observed gene expression patterns are ultimately controlled by TFs. Hence, many studies focus on CSCs reprogramming mechanisms and on the major stem cell TF networks.

I.3 Transcription factors and CSC reprogramming

TFs recognize specific DNA sequences and act as molecular switches to either activate or repress transcription of target genes. In mammals, TFs represent around 10% of all protein-coding genes (55). In 2006, Takahashi and Yamanaka evidenced the importance of TFs in cells reprogramming by inducing pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblast cultures, with a panel of defined factors: SOX2, OCT3/4, C-MYC and KLF4 (56). SOX2, OCT4 and KLF4, along with C-MYC and NANOG are some of the key TFs known to promote stemness by increasing the expression of genes involved in self-renewal and pluripotency, while suppressing genes involved in differentiation (29, 51, 57, 58).

I.3.1 SOX2 (SRY-Box Transcription Factor 2)

SOX2 is a member of the family of high-mobility group TFs that takes part in many roles throughout lineage specification, proliferation and differentiation in mammalian tissues during embryogenesis, morphogenesis and homeostasis of the foregut-derived epithelia of the esophagus, lung and trachea (59, 60). SOX2 exerts a key role maintaining the pluripotent state of embryonic stem cells (ESCs), where it is highly dosage-dependent, suggesting its function as a molecular rheostat that needs to be in balance within narrow limits with other TFs (61, 62). Small variations in SOX2 levels also modify the efficiency of reprogramming somatic cells into iPSCs (63). SOX2 is not only a master regulator in normal stem cells, it has also increasingly been associated with a CSC phenotype in several tumors, including those of the brain, breast, ovary, lung, skin, prostate, pancreas and stomach (64). Regarding GC, SOX2 role is still controversial with some authors associating its higher expression with a more aggressive phenotype, poor prognosis and worse response to therapy whereas others claim the opposite (46, 52, 64-68). Pádua *et al*. (2020) identified subpopulations of gastric CSCs in two human cell lines

– AGS and Kato III – based on the expression of SOX2 and showed that these cells display CSCs properties including increased proliferation, enhanced tumorigenesis, high ability to form gastrospheres and resistance to 5-FU (52). Supporting these results, suppression of SOX2 levels decreased cell proliferation and migration, increased apoptosis, reduced the tumorigenic potential and altered cell cycle kinetics in gastric CSCs (65). Comparable results were observed *in vivo*, where inhibition of SOX2 reduced tumor growth and decreased tumorigenicity (65, 69). Conjointly, these findings indicate that SOX2 has a pivotal role concerning several aspects of CSCs biology.

I.3.2 OCT4 (Octamer-binding Transcription Factor 4)

OCT4 is a homeodomain TF of the Pit-Oct-Unc family, involved in the regulation of ESCs stem-like properties and in cell proliferation and differentiation of adult stem cells (70-72). Several studies show that OCT4 works synergistically with SOX2, being also highly sensitive to dosage and requiring a critical amount to sustain stem-like properties, where an up- or downregulation induces divergent developmental paths (62, 73). Furthermore, both OCT4 and SOX2 regulate their own transcription by binding to the promoters of their own genes and forming interconnected autoregulatory loops (74). Abnormal expression of OCT4 has been described in several tumor types, including GC (68, 75, 76). Several studies associate its expression with a more aggressive phenotype, metastasis and poorer overall prognosis (46, 68, 75). Additionally, it is demonstrated that overexpressing OCT4 in GC cells led differentiated cancer cells to become undifferentiated and acquiring self-renewal capacity and that downregulation of OCT4 induced differentiation in GC cells (76, 77). More recently, Zhang and collaborators demonstrated the existence of a p-ERK mediated positive feedback loop between CD44 and OCT4 sustaining gastric CSCs properties (46).

I.3.3 C-MYC (MYC Proto-Oncogene, bHLH Transcription Factor)

C-MYC belongs to the MYC-family of cellular proto-oncogenes, it is frequently activated in human cancers and coordinates several biological processes in stem cells such as self-renewal, cell proliferation and differentiation, cellular growth and metabolism (78, 79). Although it facilitates the activation of the reprogramming circuitry by stimulating gene expression and proliferation, its overexpression alone is unable to induce the

transformation of normal cells into cancer cells (80, 81). C-MYC has been suggested as a potential CSC marker in several tumors such as hematopoietic malignancies, glioblastoma, neuroblastoma and prostate cancer, yet its role in GC is less studied and needs further clarification (82). In glioblastoma stem-like cells, Galardi *et al*. (2016) used a polypeptide to interfere with MYC activity and showed that this led to repression of key glioblastoma stem cells TFs including SOX2, upregulation of genes involved in differentiation and tumor suppression and deregulation of molecules implicated in growth and invasion (83). Furthermore, C-MYC has been reported to maintain self-renewal of lymphoma CSCs through cooperating with NANOG and SOX2 (84).

I.3.4 HMGA1 (High Mobility Group AT-Hook 1)

HMGA1 is a non-histone architectural chromatin protein characterized by three DNA-binding motifs (AT-hooks) that preferentially bind AT-rich DNA sequences. It is strongly expressed during embryonic development and participates in numerous cell events, including transcriptional regulation, DNA repair, chromatin remodeling and RNA processing (85-88). Several studies describe an overexpression of HMGA1 associated with poorer prognosis in numerous malignancies, including GC (89-92). Jin *et al*. (2020) showed that HMGA1 was upregulated both in GC tissues and cell lines – AGS, SGC7901, MGC803 and BGC823 – and that its overexpression facilitated cells proliferation and migration ability, as well as epithelial-mesenchymal transition (EMT) (90). Moreover, Akaboshi and collaborators reported that the role of HMGA1 in gastric cells proliferation and tumor formation is regulated via the Wnt/β-catenin pathway (89). Regarding gastric CSCs, little is known about HMGA1 functions, but a study in glioblastoma stem cells described that inhibition of endogenous HMGA1 expression decreased the expression of reprogramming TFs such as OCT4, SOX2, KLF4 and NANOG, and also stated that HMGA1 positively regulates SOX2 expression by modifying chromatin architecture at the SOX2 promoter (93). HMGA1 has also been reported to directly induce C-MYC expression and consequently facilitate GC glycolytic and neoplastic activity (91). Shah *et al*. (2012) also found a positive association between the levels of HMGA1 and the pluripotency factors NANOG, OCT4, and SOX2, and demonstrated that together with the Yamanaka factors, HMGA1 enhances cellular reprogramming of adult somatic cells to iPSCs (94).

II. AIMS

CSCs have been identified in nearly every tumor and have been proven to be responsible for poorer overall prognosis, therapy resistance and tumor recurrence. Cancer cells undergo reprogramming mechanisms that involve activation or deregulation of several core stemness signaling pathways, ultimately controlled by an elaborated transcriptional network. Thus, several current efforts aim to identify TFs that are responsible for the core reprogramming pathways and can enhance this process.

With this in mind, the main goal of this dissertation was to identify putative TFs involved in gastric CSCs reprogramming in two GC cells lines.

The specific aims were to:

- Identify candidate TFs that together with SOX2 could take part in gastric CSC reprogramming;
- Assess the impact of overexpressing the TF HMGA1 in a gastric non-CSC subpopulation;
- Optimize the co-transduction of several different TFs into a gastric non-CSC subpopulation.

III. MATERIALS AND METHODS

III.1 *In silico* screening for TFs

A comprehensive search was carried out in order to identify candidate TFs responsible for CSC identity and function in GC. The strategy was based on literature mining for TFs: 1) described in GC and/or in gastric CSCs, 2) identified as being associated with the expression of SOX2 and/or stem-like features in GC and/or other tumors and 3) described as being a SOX2 co-factor in databases as BioGRID (https://thebiogrid.org/), GeneCards (https://www.genecards.org/), Harmonizome (https://maayanlab.cloud/Harmonizome/) and The Human Protein Atlas (https://www.proteinatlas.org/), leading to the identification of 71 candidate genes (Table 3).

Table 3 – List of candidate transcription factors. Transcription factors reported to be deregulated in GC and associated with a CSC phenotype in GC and/or other tumors, as well as described to bind to SOX2.

Transcription factor	CSCs-related	SOX2 co-factor	References
ARNT2			(95, 96)
ASCL2	X		(97, 98)
ATF4			(99, 100)
ATOH1	X		(101, 102)
BARX2			(103)
BATF2			(104)
CBFB			(105)
CDX1		X	(106)
CDX2			(107)
CNOT3		X	(108-110)
C-JUN		X	(111, 112)
C-MYC	X	X	(52, 56, 78, 79)
E2F1			(113)
FOXA1	X	X	(114-118)
FOXA2	X	X	(119-122)
FOXC2	X		(123-126)
FOXM1	X	X	(127-130)
FOXO1			(131)
FOXQ1	X	X	(123, 132, 133)
GATA4/5		X	(134-136)

Table 3 – (continuation) List of candidate transcription factors. Transcription factors reported to be deregulated in GC and associated with a CSC phenotype in GC and/or other tumors, as well as described to bind to SOX2.

Transcription factor	CSCs-related	SOX2 co-factor	References
GATA6	X		(136-140)
GLI1	X	X	(46, 141-144)
GLI2	X	X	(143, 145, 146)
HES1	X		(147-149)
HMBOX1		X	(150)
HMGA1	X	X	(89-94)
HMGA2	X		(92, 150-155)
HOXA4	X		(156, 157)
HOXA5	X	X	(158-160)
HOXA10	X		(161-163)
HOXA13			(164-166)
HOXB7	X		(167-172)
HOXC10			(173-177)
HOXC6			(178, 179)
HOXC9			(180, 181)
HOXD4			(182)
HOXD9			(183, 184)
ISL1			(185-187)
KLF4	X	X	(56, 188, 189)
KLF5	X	X	(136, 190-193)
KLF16			(194)
MEIS2	X	X	(195)
MIST1	X		(196)
NANOG	X	X	(68, 197)
NF-kB	X		(198-201)
NF-YA	X	X	(202-205)
OCT1	X	X	(206-209)
OCT4	X	X	(56, 75-77)
OCT4B1		X	(210)
PRRX1			(211-213)
RUNX1			(105, 214, 215)
RUNX3		X	(216)
SALL4		X	(217-220)
SNAIL		X	(221, 222)
SOX2	X	X	(56, 61, 64-69)

Table 3 – (continuation) List of candidate transcription factors. Transcription factors reported to be deregulated in GC and associated with a CSC phenotype in GC and/or other tumors, as well as described to bind to SOX2.

Transcription factor	CSCs-related	SOX2 co-factor	References
SOX4	X	X	(223-226)
SOX9	X	X	(227-232)
SOX17			(233, 234)
Sp1		X	(235-237)
STAT3	X	X	(238-242)
TBX3		X	(243, 244)
TCF4	X		(245-247)
TCF12			(248)
TCF7L1		X	(249)
TEAD1	X		(250, 251)
TEAD2			(252, 253)
TEAD4		X	(251, 254)
YB-1	X		(125, 255-257)
ZEB1		X	(258, 259)
ZFX		X	(260, 261)
ZNF273		X	(262)

III.2 Cell Culture

For the present study, two human GC cell lines with a SORE6-GFP reporter system incorporated were used, AGS SORE6 and Kato III SORE6, previously established by our group at i3S, Porto (52), as well as HEK293T, a human embryonic kidney cell line. All cell lines were cultured in DMEM (1X) medium (Gibco, Life Technologies) without phenol red supplemented with 10% FBS (biowest) and 1% Sodium Pyruvate (Gibco, Life Technologies) and maintained in an incubator at 37 °C with an humified atmosphere containing 5% CO_2. Cells were trypsinized with 0.05% Trypsin-EDTA (1X) (Gibco, Life Technologies) once they reached a confluence of ≥ 80% and sub-cultured.

III.3 RNA extraction and Real-Time PCR

III.3.1 RNA extraction

Total RNA was isolated from cell pellets using the PureLink RNA Kit including on-column DNase treatment (Invitrogen, Thermo Fisher Scientific), following the manufacturer's instructions. Quantification and quality assessment of RNA were performed using NanoDrop ND-1000 spectrometer (V3.5.2 software) (Thermo Fisher Scientific).

III.3.2 cDNA synthesis

For this purpose 1 µg of total RNA was mixed with 1 µL of random hexamers (100 ng/µL) and DEPC-treated water (Thermo Fisher Scientific) in a final volume of 8 µL and incubated in a thermocycler (Bio-Rad) for 10 min at 65 °C. mRNA was then converted to cDNA combining 4 µL of Buffer 5X, 2 µL of DTT, 2 µL of dNTPs (10 mM), 0.3 µL of RNAseOUT (40 U/µL), 0.5 µL of SuperScript III Reverse Transcriptase (200 U/µL) and 3.2 µL of DEPC-treated water (all reagents from Invitrogen, Thermo Fisher Scientific) followed by incubation in a thermocycler for 1 h at 42 °C, followed by 10 min at 70 °C.

III.3.3 Real-Time PCR

The expression level of 26 genes was analyzed by real-time PCR. Primers (Table 4) spanning 2 adjacent exons or exon junctions to avoid genomic DNA amplification and with amplicon sizes in the range of 80 to 160 bp were designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) ruling out the possibility of any non-specific priming, and purchased from Sigma-Aldrich. Specificity of the primers was evaluated by the observation of a single peak in the melting curve. Each reaction was performed in a final volume of 20 µL containing 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.6 µL of each primer forward and reverse (10 µM), 4.8 µL of DEPC-treated water and 4 µL of cDNA diluted 1:10 in DEPC-treated water. Samples were amplified in a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following thermal cycling conditions: 40 cycles of 15 sec at 95 °C for denaturation, plus 1 min at 60 °C for annealing. Each reaction was performed in

triplicate and negative controls without cDNA were also included. Normalization of target gene expression was carried out using the endogenous housekeeping control gene 18S and data was analyzed using the $2^{-\Delta\Delta CT}$ method (263).

Table 4 – Primer sequences. Forward and reverse primer sequences used for Real-Time PCR, with the corresponding amplification product size (bp).

Gene	Primer Sequence 5'-3'	Size (bp)
ARNT2	Forward: AAGAACCGGGAGTGGATGTTG Reverse: CTGTTGCTGAAGTTGCTTGACG	117
C-JUN	Forward: AGTGAGTGACCGCGACTTTT Reverse: GATGCCTCCCGCACTCTTAC	70
CNOT3	Forward: GGCTCACGAATACCATCGACA Reverse: GCTTATCCTTGTCGCCCTTCT	108
FOXA1	Forward: AAGGGCATGAAACCAGCGAC Reverse: GCCTGAGTTCATGTTGCTGAC	89
FOXA2	Forward: ATGCACTCGGCTTCCAGTAT Reverse: GTTGCTCACGGAGGAGTAGC	108
FOXC2	Forward: CTACAGCTACATCGCGCTCATCA Reverse: ACTGGTAGATGCCGTTCAAGGTG	80
GATA6	Forward: AAGCGCGTGCCTTCATCA Reverse: CATAGCAAGTGGTCTGGGCA	156
GLI1	Forward: GAAGTCATACTCACGCCTCGAA Reverse: CAGCCAGGGAGCTTACATACAT	180
GLI2	Forward: AGCAGCAGCAACTGTCTGAGTGA Reverse: GACCTTGCTGCGCTTGTGAA	105
HES1	Forward: ACACGACACCGGATAAACCA Reverse: ATGCCGCGAGCTATCTTTCT	152
HMGA1	Forward: GTGCCAACACCTAAGAGACCT Reverse: TGTGGTGGTTTTCCGGGTC	81
HMGA2	Forward: CCCAAAGGCAGCAAAAACAA Reverse: GCCTCTTGGCCGTTTTTCTC	81
HOXA10	Forward: CCCTTCCGAGAGCAGCAAA Reverse: TCTTCCGACCACTCTTTGCC	101
HOXA5	Forward: GCGCAAGCTGCACATAAGTC Reverse: CGGAGAGGCAAAGAGCATGT	167
ISL1	Forward: CTGCTTTTCAGCAACTGGTCA Reverse: TAGGACTGGCTACCATGCTGT	123
KLF4	Forward: CAGAGGAGCCCAAGCCAAAG Reverse: TTTCTCACCTGTGTGGGTTCG	146

Table 4 – (continuation) Primer sequences. Forward and reverse primer sequences used for Real-Time PCR, with the corresponding amplification product size (bp).

Gene	Primer Sequence 5'-3'	Size (bp)
OCT1	Forward: CAAAATGGCGGACGGAGGA Reverse: GTTCATTCTTGAGTCTGCTGCTG	79
RELA	Forward: GCTGCATCCACAGTTTCCAGA Reverse: CCCCACGCTGCTCTTCTAT	129
SOX2	Forward: AACGGCTCGCCCACCTACAGC Reverse: AGTGGGAGGAAGAGGTAACC	130
SOX9	Forward: CGGAGGAAGTCGGTGAAG Reverse: CTGGGATTGCCCCGAGTGCT	165
STAT3	Forward: GAGGACTGAGCATCGAGCA Reverse: CATGTGATCTGACACCCTGAA	85
TCF4	Forward: TTTGGAAGAAGCGGCCAAGAGG Reverse: TTGGGGAGGTAGGGGCTCGT	108
TEAD2	Forward: TTTGGGGTGTGCCCAGATG Reverse: TCCTCACTGCCTTCCTCACT	90
ZNF273	Forward: AGCCTAGAAATGGGACCACTG Reverse: GCTGTGAAGTGTCCAGGCAT	85
KLF5	Forward: AAGGAGTAACCCCGATTTGG Reverse: CAGCCTTCCCAGGTACACTT	147
MEIS2	Forward: TCCAGCATCTCACACATCCG Reverse: GAAAACCTGCTCGATTTGACTGG	156

III.4 Molecular cloning

III.4.1 Transformation of competent bacteria

DNA plasmids were introduced into One Shot Stbl3 Chemically Competent *Escherichia coli*, according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific). Lentiviral vectors for overexpression of FUW-tetO-HMGA1, FUW-tetO-HOXA10, FUW-tetO-KLF4, FUW-tetO-NF-kB, FUW-tetO-OCT1 and FUW-tetO-SOX4 genes were kindly provided by Dr. Carlos Filipe Pereira, CNC/UC-Biotech, Coimbra.

III.4.2 Plasmid purification

Single *E. coli* colonies were transferred from a selective agar plate of Miller's LB Broth growth medium (Sigma-Aldrich) with 1.5% Bacto Agar (BD) supplemented with 100 µg/mL of ampicillin (Alfagene) into 10 mL of LB medium with 100 µg/mL of ampicillin and grown overnight at 37 °C under vigorous shaking. Plasmid purification was carried out following the NZYMiniprep Kit (NZYTech) according to the manufacturer's protocol. Vectors' concentration was assessed using NanoDrop 1000.

III.5 Lentiviral transduction

III.5.1 Production of viral stocks

Viral supernatants production was performed using HEK293T as a packaging cell line. HEK293T cells were cultured in 10 cm dishes at a density of $1x10^6$ cells/mL and incubated for 24 h at 37 °C and 5% CO_2. Thereafter, the designated vector of interest was co-transfected with the psPAX2 packaging plasmid and the pVSV-G envelope plasmid into HEK293T cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) in a 10 µg DNA : 15 µl lipofectamine ratio diluted in Opti-MEM medium (1X) (Gibco, Life Technologies) serum- and antibiotic-free and cells were incubated for 24 h at 37 °C and 5% CO_2. The following day, cells were exposed to fresh DMEM (1X) medium without phenol red supplemented with 10% FBS and 1% Sodium Pyruvate and incubated for 48 h at 37 °C and 5% CO_2. The lentivirus particles were collected by centrifugation at 3000 rpm for 15 min, at room temperature, and subsequent filtration by a 0.22 µm pore PES filter (Orange Scientific) to be used right away or stored at -80 °C.

III.5.2 Cell transduction

The tetracycline-inducible gene regulation system is a powerful tool that allows regulation of target gene expression. FUW-M2rtTA is a lentiviral plasmid expressing the reverse tetracycline transactivator which, in the presence of tetracycline or one of its derivatives (e.g. doxycycline) promotes transcription activation of our genes of interest.

AGS SORE6- cells were seeded in 6-well plates at a density of $5x10^5$ cells/mL in DMEM (1X) medium without phenol red, supplemented with 10% FBS and 1% Sodium Pyruvate, and incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2.

For HMGA1 overexpression, cells were transduced with the FUW-tetO-HMGA1 expression vector in a 1 mL of fresh medium:1 mL of FUW-M2rtTA viral supernatant:1 mL of FUW-tetO-HMGA1 viral supernatant ratio, for 48 h at 37 °C and 5% CO_2. Then, cells were exposed to fresh medium containing 2 µg/mL of doxycycline for 48 h at 37 °C and 5% CO_2. Cells with 1 mL of FUW-M2rtTA viral supernatant:2 mL of fresh medium were used as a negative control.

To assess the efficiency of transducing 5 different TFs at once, cells were exposed to viral supernatants diluted in fresh medium. Different dilutions and incubation time periods were tested, namely: a dilution ratio of 1 mL of fresh medium:1 mL of FUW-M2rtTA:1/5 mL of each TF viral supernatant and a ratio of 1 mL of FUW-M2rtTA:1/5 mL of each TF viral supernatant, with differential exposures of 24 h, 48 h and 24 h followed by a new infection with the TFs cocktail for additional 24 h (double shot). Cells incubated with 1 mL of FUW-M2rtTA:1 mL of fresh medium or 1 mL of FUW-M2rtTA :2 mL of fresh medium were used as a negative control. After incubation with the TFs cocktail, fresh medium with 2 µg/mL doxycycline was added and cells were incubated for 48 h at 37 °C and 5% CO_2. GFP expression was observed using ZOE Fluorescence Cell Imager (Bio-Rad).

III.6 Flow Cytometry and Fluorescence-Activated Cell Sorting

III.6.1 Flow Cytometry

Transduced cells were collected by trypsinization and centrifuged at 1200 rpm for 5 min at room temperature. Cell pellets were then washed with PBS (grisp) and resuspended in PBS with 10% FBS. Flow cytometry was performed on Accuri C6 flow cytometer (BD Biosciences) for GFP expression analysis. Data was analyzed using FlowJo software (Version 7.6.1).

III.6.2 Fluorescence-Activated Cell Sorting

Transduced AGS SORE6 cell line samples were handled as described previously in 4.1. Cells transduced with only FUW-M2rtTA were used as a negative control for gate assessment, while cells transduced with FUW-tetO-HMGA1 were sorted using FACS Aria II Cell Sorter flow cytometer (BD Bioscience). Positive and negative cells for GFP expression – HMGA1 GFP+ and HMGA1 GFP-, respectively - were sorted out and seeded in 6-well plates at a density of $5x10^3$ to $1x10^5$ cells/mL and cultured in DMEM (1X) without phenol red supplemented with 10% FBS, 1% Sodium Piruvate and 1% Pen Strep (Gibco, Life Technologies) in a humidified incubator at 37°C and 5% CO_2. Data was analyzed using FlowJo software.

III.7 Protein extraction and Western Blot

III.7.1 Protein extraction and quantification

Protein extraction from whole cell extracts was carried out by resuspending cell pellets 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 1 mM PMSF, 1 mM Na_3VO_4 and the Complete protease inhibitor cocktail (Roche) followed by incubation on ice for 15 min and centrifugation of lysates at 16000 g at 4 ℃ for 15 min. The supernatant containing the purified protein extract was then collected and protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

III.7.2 Western Blot

For western blot analysis a total of 30 µg of total protein extract were fractionated by standard SDS-PAGE using the Precision Plus Protein Standard Dual Color protein marker (Bio-Rad) and transferred into a nitrocellulose membrane (Amersham, GE Healthcare). After staining with ponceau red (Sigma-Aldrich) to assess the efficiency of the process, membranes were washed with TBS 1% Tween-20 (Sigma-Aldrich) and blocked for 1 h at room temperature with either 5% non-fat milk diluted in TBS 1% Tween-20 or 5% BSA (Sigma-Aldrich) in TBS 1% Tween-20. After, membranes were incubated with

primary antibodies for SOX2, C-MYC and *β*-actin (loading control) (Table 5) at 4 °C overnight. Subsequently, blots were washed with TBS 1% Tween-20 and incubated with the correspondent HRP-conjugated secondary antibody (Table 5) at room temperature for 1 h. Signal detection was assessed using the ECL detection kit (Amersham, GE Healthcare).

Table 5 – Western blot antibodies. Primary and secondary antibodies used for western blot analysis, with the corresponding target, type, weight (kDa), dilution and manufacturer.

Primary Antibodies				
Target	**Type**	**Molecular Weight (kDa)**	**Dilution**	**Manufacturer**
SOX2	Rabbit monoclonal	34	1:500 Milk	Cell Marque
C-MYC	Rabbit monoclonal	65	1:600 BSA	Cell Signaling
β-actin	Mouse monoclonal	42-44	1:900 Milk	Santa Cruz
Secondary Antibodies				
Rabbit igG (HRP)	Goat anti-rabbit	-	1:10000 TBS-Tween-20	Cell Signaling
Mouse igG (HRP)	Goat anti-mouse	-	1:1500 Milk	Santa Cruz

III.8 BrdU labeling for proliferation analysis

AGS SORE6- HMGA1 GFP+ cells were seeded in 6-well plates at a density of $5x10^5$ cells/mL in DMEM (1X) without phenol red and supplemented with 10% FBS and 1% Sodium Pyruvate and incubated for 24 h at 37 °C and 5% CO_2. Thereafter, cells were exposed to BrdU (Roche) diluted in medium at a 1:1000 ratio for 1 h at 37 °C. After incubation, cell pellets were collected by trypsinization, washed with PBS and fixed in 1 mL of ice-cold methanol (Chem-Lab) for 30 min. After washing with PBS, cells were permeabilized with 1 mL of HCl 4M (Mallinckrodt Baker) for 20 min at room temperature and again washed with PBS. After a blocking step in PBS with 0.5% Tween-20 and 0.05% BSA for 10 min, cells were incubated with a monoclonal mouse primary antibody anti-BrdU (Cell Signaling) diluted 1:20 µL in the blocking solution, for 1 h at room temperature. Cells were then washed with PBS and incubated with the polyclonal rabbit anti-mouse secondary antibody labelled with FITC (Dako) diluted 1:150 µL for 30 min at room temperature protected from light. After a final washing step, the pellet was

resuspended in 500 µL of PBS and the percentage of BrdU labeled positive cells was evaluated using the FACS Aria II flow cytometer. The data was analyzed using FlowJo software.

III.9 Sulforhodamine B assay for cytotoxicity analysis

AGS SORE6- HMGA1 GFP+ cells were plated in 96-well plates at a density of $2{,}5x10^4$ cells/mL in DMEM (1X) without phenol red and supplemented with 10% FBS and 1% Sodium Pyruvate and were incubated for 24 h at 37 °C and 5% CO_2. The day after, 5-FU (Sigma-Aldrich) was administered in a dose (4.25 µg/mL) corresponding to the respective IC_{50}, previously calculated by our group, and cells were incubated for 48 h. Then, cells were fixed with cold 10% (w/v) TCA (Merck Millipore), incubated on ice for 1 h, washed with dH_2O and let to air-dry at room temperature. The plates were then stained with 0.4% (w/v) of Sulforhodamine B (Sigma-Aldrich) for 30 min, washed with 1% (w/v) of acetic acid (Sigma-Aldrich) and allowed to air-dry, after what the dye was solubilized with 10 mM Tris-Base pH=10.5 (Sigma-Aldrich). The respective absorbances at 510 nm were measured using the Synergy Microplate Reader (Biotek).

IV. RESULTS

IV.1 TFs expression analysis by Real-Time PCR

An *in silico* screening for TFs that could potentially be responsible for inducing gastric CSC properties revealed 71 possible candidates. From this pool, mRNA expression of 26 genes was analyzed by Real-Time PCR. When compared to the AGS SORE6- population, AGS SORE6+ cells revealed an enrichment, although not statistically significant, of KLF5, STAT3, RELA, HMGA1, GATA6, ZNF273, OCT1, KLF4 and HOXA10 (Figure 2a). Kato III SORE6+ cells showed increased levels, also not statistically significant, of C-JUN, RELA, GLI1, CNOT3, KLF4, KLF5, HMGA1, HMGA2, HES1, GATA6 and FOXC2, when compared to Kato III SORE6- cells (Figure 2b). Altogether, both GC cell lines share increased mRNA expression levels of 5 genes: KLF5, RELA, HMGA1, GATA6 and KLF4.

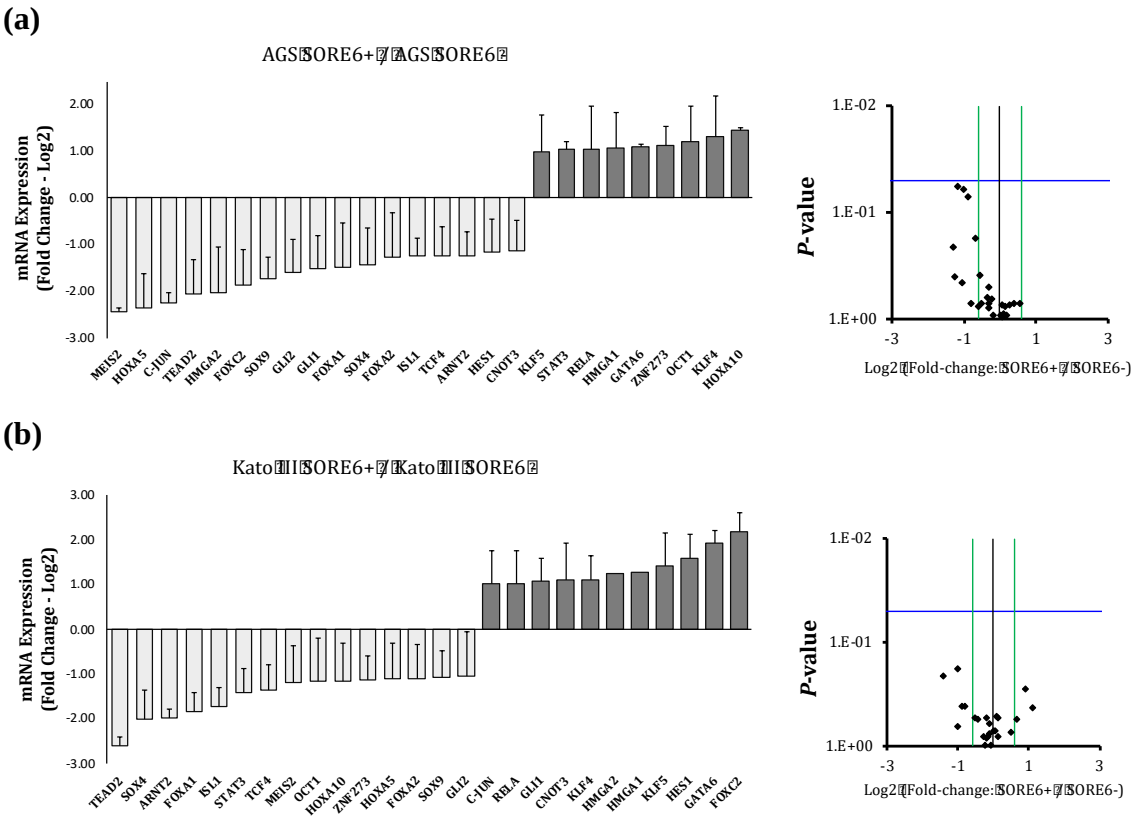

Figure 2 – **Real-Time PCR analysis.** mRNA levels of 27 transcription factor genes present on a) AGS SORE6 and b) Kato III SORE6 cell lines, normalized to 18S expression. Results are represented as fold change (up- or down-regulation) compared to SORE6+ and SORE6- cells, and include mean ± SD, except for HMGA1 and HMGA2 genes on Kato III SORE6 cell line (n=1). *P*-values were also evaluated.

IV.2 HMGA1 overexpression in AGS SORE6^{-} cells

From the five TFs that were increased in both cell lines, although only marginally increased, HMGA1 caught our attention since its expression has been linked to CSC features such as increased cell proliferation and migration ability, as well as EMT (89, 90, 92). Furthermore, it has been associated with core TFs involved in CSC reprogramming, namely SOX2 and OCT4 and when added to Yamanaka's cocktail it enhances the reprogramming of iPSCs (93, 94), therefore we thought it was a strong candidate for cellular reprogramming. We assessed the expression of HMGA1 in AGS SORE6 cells by western blot analysis and saw that HMGA1 was expressed in AGS SORE6+ cells, and absent in AGS SORE6- cells (Figure 3) also at the protein level.

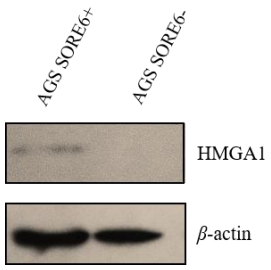

Figure 3 – HMGA1 expression in AGS SORE6 cells. Western blot analysis of HMGA1 expression in AGS SORE6+ and AGS SORE6- cells, respectively. β-actin was used as an internal control.

With this in mind, we decided to assess the impact of overexpressing HMGA1 in AGS SORE6- cells. For this purpose, cells were transduced with an expression vector containing the HMGA1 gene for 48 h, and later gene expression was activated with doxycycline for another 48 h.

AGS SORE6 cells have an incorporated lentiviral reporter construct – SORE6-GFP – to which SOX2 and/or OCT4 can bind to activate the expression of the reporter gene GFP (51, 52). Importantly, the system requires a significant level of expression to be activated and GFP intensity varies according to the expression levels of SOX2 and OCT4 (51). Based on this, and since AGS SORE6- cells show no signal for GFP fluorescence, we used the SORE6-GFP reporter system as a readout of cellular reprogramming (Figure 4a). Therefore, transduced cells were collected and analyzed by flow cytometry for assessing GFP levels (Figure 4b). Interestingly, a total of 2.0% of the cells were able to activate the reporter system and exhibited a positive signal for GFP

fluorescence (Figure 4b). The efficiency of transduction was assessed by Real-Time PCR (Figure 4c).

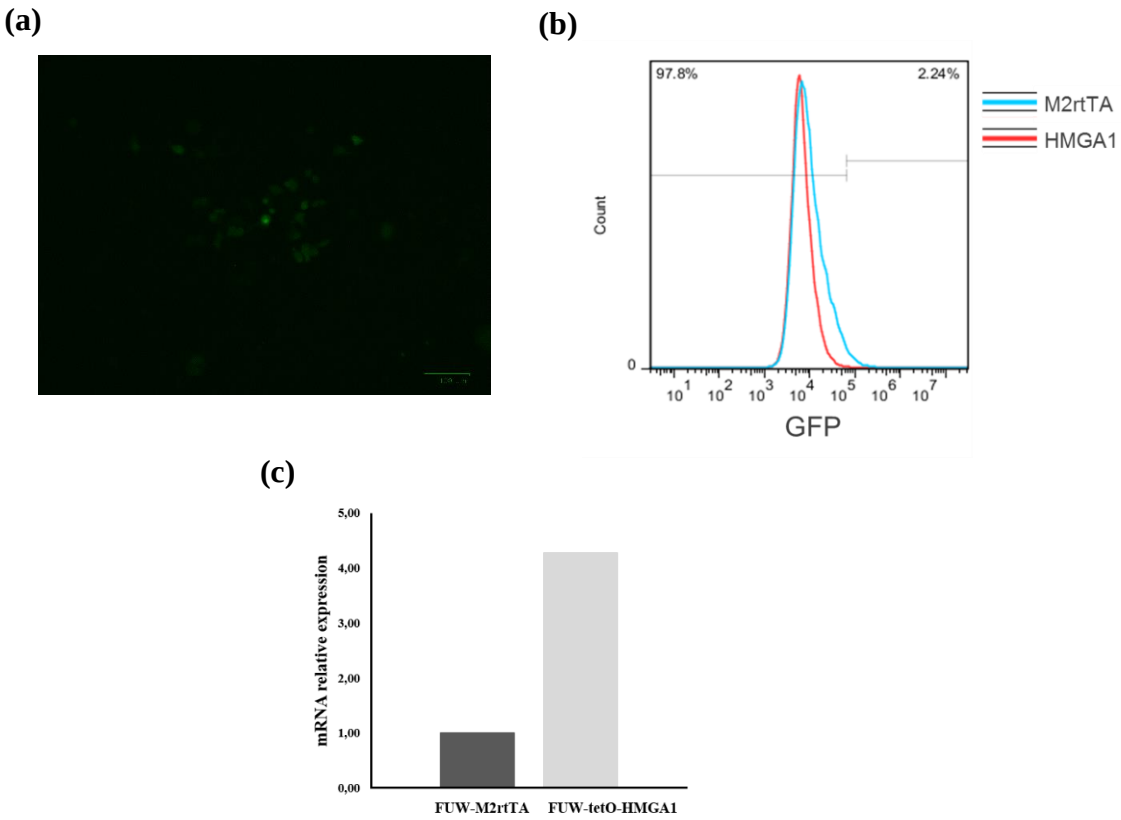

Figure 4 – HMGA1 overexpression on AGS SORE6- cells. (a) Fluorescence image showing activation of SORE6-GFP reporter system in AGS SORE6- cells transduced with the FUW-tetO-HMGA1 lentiviral vector. (b) Flow cytometry analysis of AGS SORE6- cells infected with FUW-M2rtTA or FUW-tetO-HMGA1 supernatants; cells with FUW-M2rtTA were used as gating control. (c) Real-Time PCR analysis to assess the efficiency of transduction FUW-tetO-HMGA1 into AGS SORE6- cells.

IV.3 HMGA1 overexpression upregulates key stemness transcription factors SOX2 and C-MYC

The identification of cellular subpopulations expressing GFP within the AGS SORE6- cells transduced with HMGA1 (AGS SORE6- HMGA1 GFP+) led to its isolation by FACS (Figure 5a). AGS SORE6- HMGA1 GFP+ cells showed an improved growth in terms of cell density throughout the days following sorting, when compared to HMGA1 GFP- cells (Figure 5b). Pádua *et al.* (2020) previously reported that AGS SORE6+ cells with a stem cell phenotype have increased expression levels of SOX2 and C-MYC, compared to no expression detected in AGS SORE6- (52). Thus, in order to evaluate if AGS SORE6- HMGA1 GFP+ cells share stem-like properties with AGS SORE6+ cells, we decided to assess the protein levels of both TFs by western blot analysis. HMGA1 GFP+ cells exhibited a significant enrichment of both SOX2 and C-MYC when compared to the HMGA1 GFP- cell population (Figure 5c).

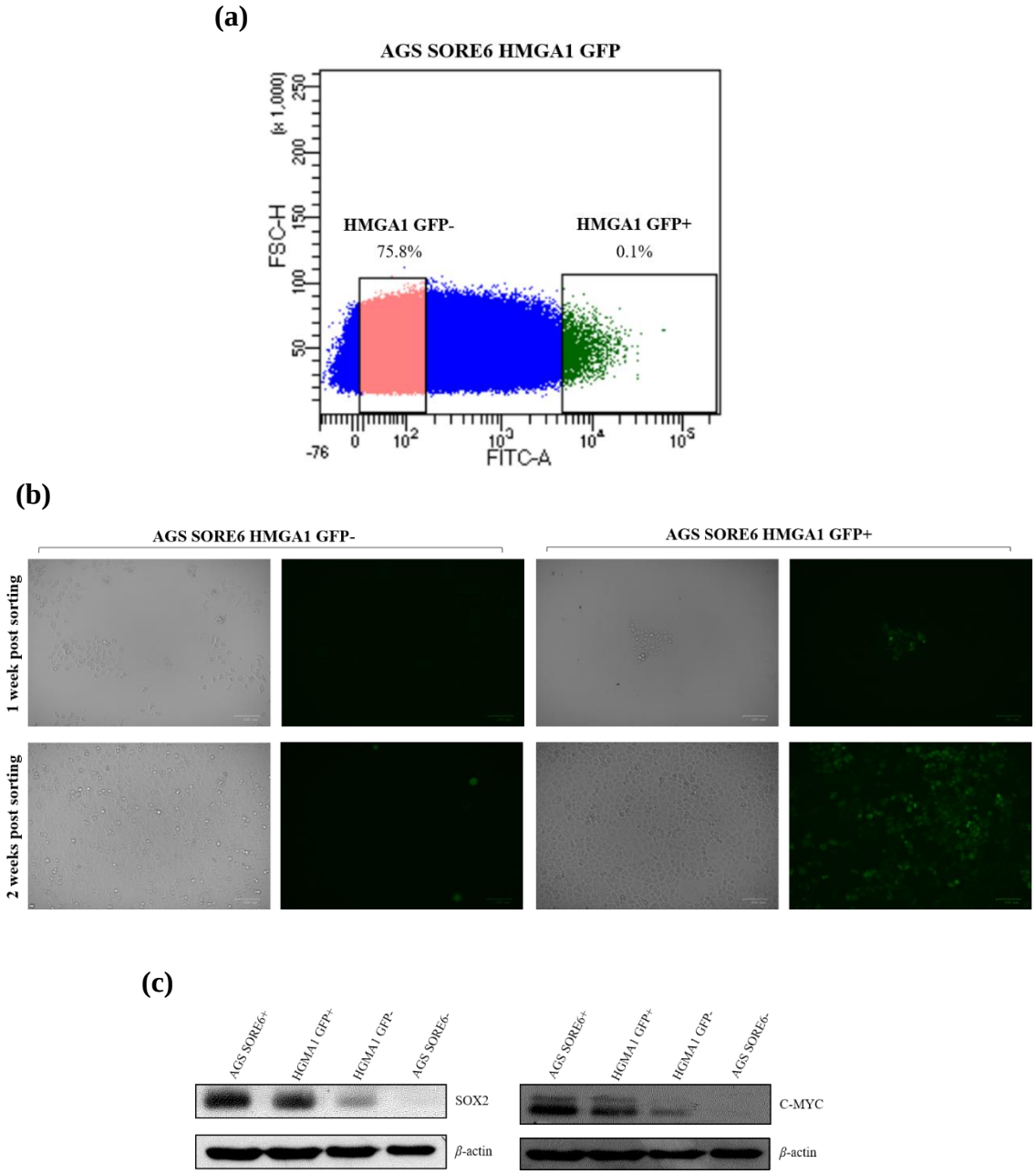

Figure 5 – HMGA1 GFP+ and HMGA1 GFP- cell populations. (a) FACS analysis with gate selection of two populations in AGS SORE6 cell line transduced with the HMGA1 expression vector, one population expressing GFP (HMGA1 GFP+) - 0.1% of cells - and another without GFP expression (HMGA1 GFP-) - 75.8% of cells. (b) HMGA1 GFP+ and HMGA1 GFP- cell populations 1 week and 2 weeks post sorting. (c) western blot analysis of SOX2 and C-MYC proteins present on AGS SORE6+, HMGA1 GFP+, HMGA1 GFP- and AGS SORE6- cell populations, respectively. *β*-actin was used as an internal control.

IV.4 Cell proliferation analysis

The proliferation rate can be measured with the thymidine analog BrdU, which is incorporated by cells into the newly synthetized DNA strands. Since AGS SORE6+ cells have a greater ability to proliferate compared to AGS SORE6- cells (52), we decided to assess if there were similarities between the subpopulations and evaluate the proliferation of HMGA1 GFP+ and HMGA1 GFP- cells through a BrdU assay. The results showed that HMGA1 GFP+ cells are significantly more proliferative when compared to HMGA1 GFP- cells (Figure 6). About 44.4% of AGS SORE6- HMGA1 GFP+ cells were positive for green fluorescence, compared to 33.1% for AGS SORE6- HMGA1 GFP- cells.

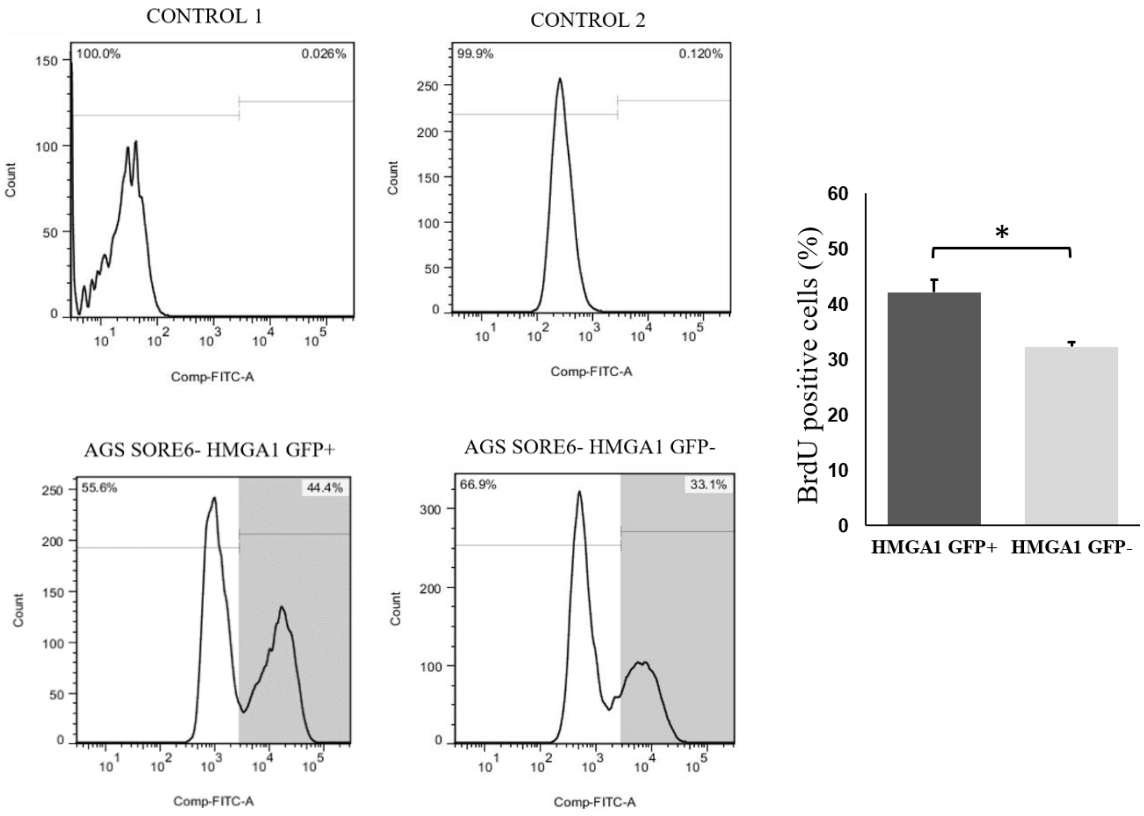

Figure 6 – BrdU assay for cell proliferation analysis trough FACS. Control 1 was used for primary antibody control, while control 2 was used for the secondary antibody and gating control. (a) About 44.4% and 33.1% of AGS SORE6- HMGA1 GFP+ and AGS SORE6- HMGA1 GFP- cells, respectively, were positive for fluorescence. Results are mean ± SD. Significant differences (*p≤0.05).

IV.5 Cytotoxicity analysis after incubation with 5-FU

Apart from a higher proliferation rate and expression of stem cells core TFs, AGS SORE6+ cells also show an increased resistance to the therapeutic drug 5-FU in comparison with AGS SORE6- cells (52). Therefore, the cytotoxicity of 5-FU on both AGS SORE6 HMGA1 GFP+ and AGS SORE6 HMGA1 GFP- cells was evaluated using the Sulforhodamine B assay, used for cell density determination based on the measurement of cellular protein content. The results show that there is no significant difference in the sensitivity to 5-FU concerning AGS SORE6 HMGA1 GFP cells, with around 80% of both subpopulations dead after 48 h of treatment (Figure 7).

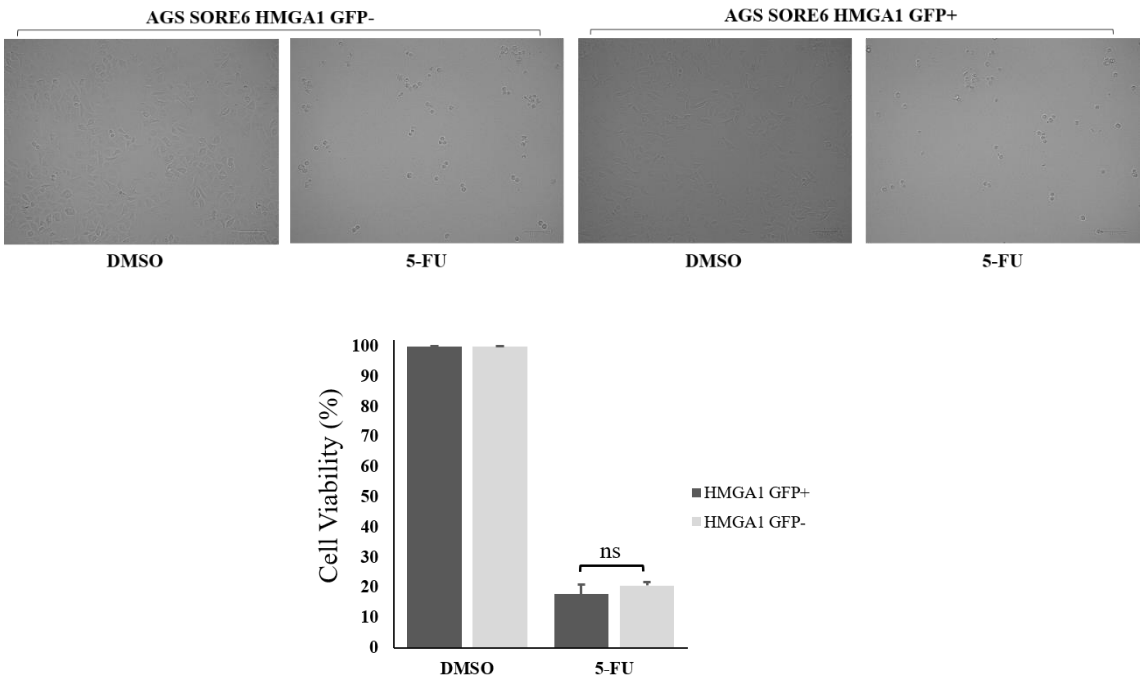

Figure 7 – Sulforhodamine B assay for cell cytotoxicity analysis. Results show that after 48 h of treatment with 5-FU, the percentage of cell viability was about 17.0% and 21.4% for AGS SORE6- HMGA1 GFP+ and AGS SORE6- HMGA1 GFP- cells, respectively. Cells treated with DMSO were used as control. Results are mean ± SD. ns- not significant.

IV.6 TFs combinations as a cellular reprogramming strategy

In vitro cellular reprogramming is a slow and ineffective process, with an estimated efficiency in human cells of around 0.01%. Also, an efficient and more complete reprogramming is usually achieved by combining a cocktail of several TFs. Taking this into account, we decided to try an approach where we introduced a higher number of TFs into cells and so we started by optimizing this assay. For this purpose, co-transduction of five different TFs into AGS SORE6- cells was tested using two distinct dilutions of viral supernatants and different incubation time periods of 24 h, 48 h and 24 h followed by a new infection with the TFs cocktail for additional 24 h (double shot). Expression levels of overexpressed genes were assessed by Real-Time PCR and the results reveal that a dilution of 1:3 – 1 mL of fresh medium : 1 mL of FUW-M2rtTA : 1/5 mL of each TF viral supernatant (FUW-SOX4, FUW-KLF4, FUW-RELA, FUW-HOXA10 and FUW-OCT1) – for 48 h seems to be the best option (Figure 8). SOX2 relative expression was also analyzed to assess if introducing new TFs had any consequence on its endogenous levels. Interestingly, mRNA levels of SOX2 presented a slight increase, however these results need further validation.

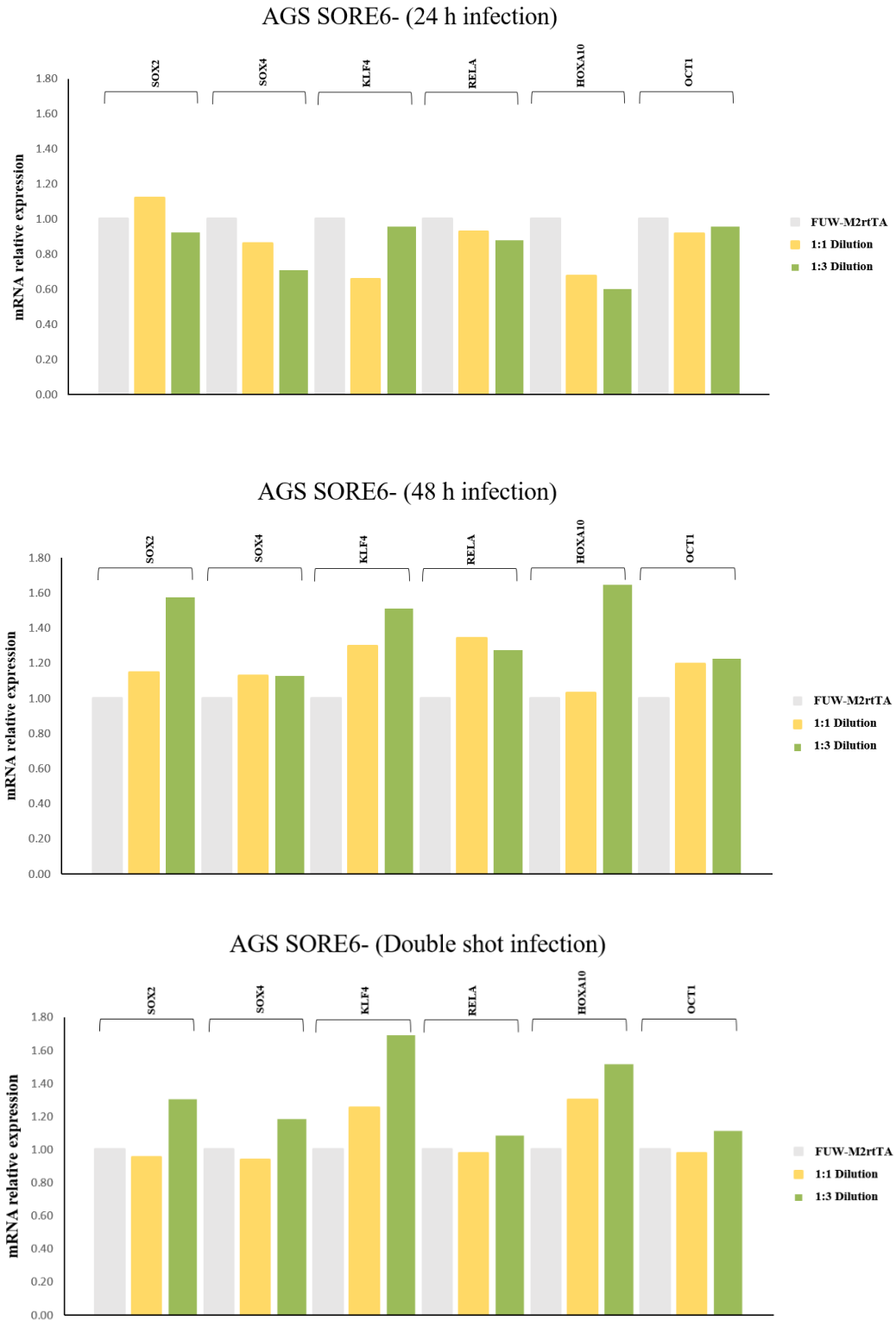

Figure 8 – Co-transduction of five different transcription factors. mRNA expression levels of five genes introduced in AGS SORE6- cells (SOX4, KLF4, RELA, HOXA10 and OCT1) using two distinct dilutions of viral supernatants and different incubation times of 24 h, 48 h and 24 h followed by a new infection with the TFs cocktail for additional 24 h (double shot), respectively. SOX2 expression for each assay was also analyzed. Cells incubated with FUW-M2rtTA were used as control.

V. DISCUSSION

GC remains a significant health problem worldwide with most patients being diagnosed late, which leads to poorer overall prognosis and tumor relapse. A small subpopulation of cancer cells – CSCs – is thought to be the origin of tumorigenesis and the main cause for therapy resistance and disease relapse (17). These cells display activation and deregulation of several core stemness signaling pathways controlled by a complex network of TFs. Thus, identifying this network is crucial and provides new tools for cancer prognosis and treatment.

In the present study we performed a comprehensive *in silico* screening for TFs described as being deregulated in GC and/or in gastric CSCs, as well as being related to the stemness factor SOX2 in GC and other tumors. From 71 possible candidates we selected a pool of 26 genes and further analyzed their mRNA expression levels through Real-Time PCR in two GC cell lines previously established by our group, AGS SORE6 and Kato III SORE6 (52). These cells have an incorporated reporter construct, SORE6-GFP, that drives expression of GFP based on the expression of SOX2 and OCT4. The results revealed that both AGS and Kato III SORE6+ cells share an upregulation of 5 TFs: KLF4, KLF5, GATA6, RELA and HMGA1. KLF4 is a well-known core stemness TF originally used by Yamanaka in the reprogramming of somatic cells (56). Although several studies describe KLF4 as being downregulated in GC and negatively associate its low expression with worst patient overall survival (188, 189, 264, 265), our results show that KLF4 levels are increased in gastric CSCs subpopulations. KLF4 is also described to be upregulated in CSCs of several tumor types, including osteosarcoma, colon and breast cancer (266-268). Studies in iPSCs suggest that KLF4 acts as a pioneer promoting transcriptional upregulation of stem cells genes, but its ability to activate some *loci* is dependent on preexisting chromatin accessibility or DNA methylation status. KLF4 binding is not always sufficient for gene activation, suggesting the requirement of additional TFs and coregulators, such as NANOG and OCT4 (269-275). The role of KLF5 is less studied in GC. Chen *et al*. (2020) reported that KLF5 is upregulated and positively associated with GC TNM staging, tumor size, metastasis and poor prognosis (191). Another study correlated an increased expression of KLF5, together with GATA6, to a higher proliferation rate of GC cells, implying these TFs may be responsible for promoting GC (136). More recently, a potential role of KLF5 on stem cell maintenance starts to emerge, mainly linked to chemotherapeutic drug resistance (276-278). In ovarian

cancer KLF5 is highly expressed and enhances drug resistance of stem-like cells to cisplatin and paclitaxel, mainly by upregulating survivin, an inhibitor of apoptosis (277). Also, in triple-negative breast cancer, metformin suppresses CSCs by targeting KLF5 for degradation (278). The role of GATA6 in GC is still controversial, with some studies reporting its downregulation and silencing, while others describe its upregulation (136-138, 279, 280). Our results show that GATA6 is enriched in gastric CSCs. Likewise, it has been reported that GATA6 enhances stemness and promotes self-renewal of colonic CSCs (139, 140, 281). Lai *et al*. (2019) reported that overexpression of GATA6 in colorectal cancer cells significantly improved their stemness properties and self-renewal, increased the levels of several stem cell marker genes including C-MYC, NANOG, OCT4, CD44, CD133 and ALDH1, and also enhanced *in vivo* tumorigenicity (281). RELA, also known as p65, encodes a subunit of the NF-κB complex, frequently upregulated and associated with worst prognosis in GC (199, 282-286). Also, it has been implicated as a relapse prediction marker for the chemotherapeutic efficacy of 5-FU in GC, possibly acting by compensating the function of p53 (287). Concerning CSCs there is ample evidence of the activation of NF-κB on several malignancies, including glioblastoma, breast, ovary, prostate, lung and pancreatic cancer (288-292). Glioblastoma CSCs exhibit a constitutive activation of NF-κB, mainly due to RELA overexpression, which deregulates several genes of the Notch signaling pathway, important for cell fate determination, survival, proliferation and maintenance of stem cells (291). NF-κB is also overexpressed in pancreatic CSCs, working together with SOX9 to improve cells migration and invasion ability and to maintain their stemness phenotype (293). Zakaria *et al*. (2018) evaluated the impact of inhibiting NF-κB on lung CSCs and revealed that it effectively reduced stemness, self-renewal and migration capacity, as well as downregulated the expression of stem cell TFs – SOX2, NANOG, and OCT4 – and genes involved in EMT and apoptosis resistance (292). High levels of HMGA1 are found in fast proliferating tissues and neoplastic cells, with absent or low expression levels in normal differentiated adult tissues (294). Further studies uncovered high levels of HMGA1 in several human tumors from diverse tissues, including those of the lung, pancreas, breast, prostate, liver and colon (295-300). HMGA1 is also often overexpressed in GC and is associated with high proliferation, increased migration ability and EMT (89, 90). A study using human ESCs shows that HMGA1 controls dedifferentiation by modulating the expression of the stem cells genes SOX2 and C-MYC (94). Furthermore, when added to the original Yamanaka's cocktail, HMGA1 enhances the cellular

reprogramming of somatic cells resulting in significant larger cell colonies, indicating that HMGA1 improves the reprogramming rate, stem cell survival and proliferation throughout iPSC generation (94). HMGA1 is also important in CSCs. Puca *et al*. (2014) demonstrated that HMGA1 is enriched in colon CSCs and that its silencing drastically reduces cell proliferation and increases stem cell quiescence, while decreasing self-renewal and sphere-forming capacity (301). Furthermore, a study in glioblastoma stem cells associated HMGA1 expression with the expression of key reprogramming factors such as OCT4, SOX2, KLF4 and NANOG, and stated that HMGA1 directly regulates SOX2 expression (93). Also in glioblastoma stem cells, HMGA1 knockdown attenuated self-renewal and sphere forming efficiency, increased cells sensitivity to the therapeutic drug temozolomide and led to reduced tumor initiation ability *in vivo* (302). Altogether these findings suggest a central role for HMGA1 in neoplastic transformation and gastric CSC reprogramming. Hence, we first validated HMGA1 increased expression on AGS SORE6+ cells by western blot analysis and later proceeded to assess the impact of overexpressing this TF on AGS SORE6- cells, using a lentiviral FUW-tetO-HMGA1 vector. GFP expression, that reflects the expression of SOX2 and OCT4, was used as a readout of a possible reprogramming. Our results disclose that a small percentage of transduced cells succeeded in activating the SORE6-GFP system and we were able to isolate a subpopulation of cells positive for GFP fluorescence (AGS SORE6- HMGA1 GFP+ cells). We confirmed by western blot that these cells, positive for GFP, show an upregulation of SOX2, compared to absence of expression of this TF in AGS SORE6- cells and a reduced expression in HMGA1 GFP- cells. Consistent with this, Lopez-Bertoni *et al*. (2016) demonstrated that HMGA1 directly regulates and activates SOX2 by modifying chromatin structure at its promoter, in glioblastoma stem cells (93). Since the SORE6-GFP reporter system requires a minimum amount of SOX2 to activate GFP (51), the reduced levels observed on AGS SORE6- HMGA1 GFP- cells might not have been sufficient to activate it.

Several studies place SOX2 at the top of the hierarchy of cell reprogramming and associate its upregulation with CSC characteristics. Our group previously isolated and characterized AGS SORE6+ cells that have stem properties related to SOX2 expression and showed these cells had a higher proliferation rate and increased expression of C-MYC, compared to AGS SORE6- cells (52). Furthermore, HMGA1 has been reported to induce C-MYC expression in AGS cells by direct interaction with its promoter, while HMGA1 knockdown consequently suppresses C-MYC mRNA expression (91).

Consistent with these studies, we confirmed that AGS SORE6- HMGA1 GFP+ cells have a similar expression profile as AGS SORE6+ cells, showing an upregulation of C-MYC, compared to lesser levels on HMGA1 GFP- cells and no observed expression on AGS SORE6- cells. These results seem to parallel those obtained for SOX2 expression levels, which may indicate that either HMGA1 and/or SOX2 might possibly be regulating C-MYC expression. Interestingly, Akaboshi *et al*. (2009) demonstrated that HMGA1 can also be induced by C-MYC, suggesting a positive loop between both TFs (89). Regarding proliferation ability, AGS SORE6- HMGA1 GFP+ cells are significantly more proliferative when compared to HMGA1 GFP- cells. The C-MYC oncoprotein has been widely studied for its role in proliferation and metabolism of both normal and neoplastic cells. A gene ontology analysis revealed that C-MYC targets in ESCs comprise numerous regulators of metabolic processes including translation, RNA splicing, cell cycle and energy production, while SOX2, OCT4 and KLF4 targets are mainly transcriptional regulators of development and differentiation (303). Noteworthy, an HMGA1-C-MYC axis has been implicated in the process regulation of glycolysis in GC, where HMGA1 knockdown showed prominent effects on glucose uptake, lactate production, extracellular acidification ratio, cell proliferation and cell invasion (91).

Another crucial property of CSCs is the enhanced resistance to therapeutic drugs. Our group has previously reported that AGS SORE6+ cells presented an increased resistance to 5-FU, and that transiently overexpressing SOX2 in AGS SORE6- cells made them more resistant to 5-FU, suggesting a determinant role for SOX2 (52). However, in this study we did not find any significant difference between AGS SORE6- HMGA1 GFP+ and HMGA1 GFP- cells resistance to this drug. Since HMGA1 GFP+ and HMGA1 GFP- cells do not exhibit such a striking difference in SOX2 levels as seen in AGS SORE6+ when compared to AGS SORE6- cells, the differences in HMGA1 levels may not be sufficient to activate the required levels of SOX2 and more TFs need to be added for a more efficient reprogramming.

Recently, iPSCs have been generated from a range of human cancer cell lines in an attempt to recapitulate the development of cancer and understand the underlying mechanisms involved. Several authors have been using an approach consisting of retroviral transduction of Yamanaka's TFs – OCT3/4, SOX2, KLF4 and C-MYC – into cancer cell lines from retinoblastoma, pancreatic, liver, gastric and colorectal cancers (312-316). These transduced cells exhibit CSC properties, including increased expressions of CSC markers, colony formation, increased chemoresistance and

tumorigenic potential *in vivo* (304-308). The reprogramming of cancer cells using iPSC technology arises as a novel approach for the study of CSC-related genes, providing a useful model for studying the genes involved in generation of CSCs before and after reprogramming, as well as elucidating the mechanisms underlying cancer initiation and progression. With that in mind, we proceeded to optimize the co-transduction of five different TFs – SOX4, KLF4, RELA, HOXA10 and OCT1 – on the AGS SORE6- cell line, testing different dilution ratios and incubation times. The results showed that all TFs successfully entered cells and, interestingly, the combination seems to influence SOX2 expression levels. However, further validation of these results is necessary.

VI. CONCLUSION

The main goal of this study was to provide a better understanding of gastric CSC transcriptional network. The analysis of the literature revealed a comprehensive list of TFs deregulated in GC and CSCs, from which a set of 26 genes were analyzed in two GC cell lines, AGS SORE6 and Kato III SORE6. Five genes were found to be overexpressed in subpopulations with a CSCs phenotype isolated from both cell lines: KLF4, KLF5, GATA6, RELA and HMGA1. Overexpressing HMGA1 in AGS SORE6- subpopulation, known to not having CSC properties, led to the isolation of putative reprogrammed cells with increased expression of the stem cell TFs SOX2 and C-MYC, as well as improved proliferation ability. However, no significant results were obtained for 5-FU sensitivity, suggesting the difference in HMGA1 levels is not sufficient to determine differences in the response to the drug and more TFs may be needed for a more efficient reprogramming.

Overall, our results suggest that HMGA1 might play a key role and act as a valuable player in gastric CSC reprogramming, possibly through a regulatory HMGA1/SOX2/C-MYC axis.

VII. FUTURE PERSPECTIVES

Our current results seem promising, however additional studies need to be done to further support obtained data. Evaluating other CSCs features apart from proliferation and 5-FU resistance such as asymmetric division, gastrosphere formation, chemoresistance to other therapeutic drugs and *in vivo* tumorigenesis ability in nude mice could give more insights about the depth of HMGA1 induced reprogramming. Also, to disclose the regulatory role of a possible HMGA1/SOX2/C-MYC axis, co-immunoprecipitation and luciferase reporter assays are mandatory.

The role of other TFs found to be overexpressed in AGS SORE6 and Kato III SORE6 CSCs should also be farther studied.

Further optimization of the protocol used for co-transducing several transcription factors into GC cells is also required. Other combinations of lentiviral vectors are necessary for a steadier validation of the process and could unveil the importance of other key stem regulators.

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