Original Article

SOX9 knockout decreases stemness properties in colorectal cancer cells

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Background: Colorectal cancer (CRC) is a leading cause of death worldwide. SRY-box transcription factor 9 (SOX9) participates in organogenesis and cell differentiation in normal tissues but has been involved in carcinogenesis development. Cancer stem cells (CSCs) are a small population of cells present in solid tumors that contribute to increased tumor heterogeneity, metastasis, chemoresistance, and relapse. CSCs have properties such as self-renewal and differentiation, which can be modulated by many factors. Currently, the role of SOX9 in the maintenance of the stem phenotype has not been well elucidated, thus, in this work we evaluated the effect of the absence of SOX9 in the stem phenotype of CRC cells.

Methods: We knockout (KO) *SOX9* in the undifferentiated CRC cell line HCT116 and evaluated their stemness properties using sphere formation assay, differentiation assay, and immunophenotyping.

Results: *SOX9-KO* affected the epithelial morphology of HCT116 cells and stemness characteristics such as its pluripotency signature with the increase of *SOX2* as a compensatory mechanism to induce SOX9 expression, the increase of *KLF4* as a differentiation feature, as well as the inhibition of the stem cell markers CD44 and CD73. In addition, *SOX9-KO* cells gain the epithelial-mesenchymal transition (EMT) phenotype with a significant upregulation of *CDH2*. Furthermore, our results showed a remarkable effect on first- and second-sphere formation, being *SOX9-KO* cells less capable of forming high-size-resistant spheres. Nevertheless, CSCs surface markers were not affected during the differentiation assay.

Conclusions: Collectively, our findings supply evidence that SOX9 promotes the maintenance of stemness properties in CRC-CSCs.

Keywords: Colorectal cancer (CRC); cancer stem cells; cancer stem cells (CSCs); SRY-box transcription factor 9 (SOX9); CRISPR-Cas9; stemness

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Introduction

Colorectal cancer (CRC) is one of the most relevant malignancies around the globe. It accounts for over 916,000 deaths being the 2nd most deadly cancer, affecting 1,931,590 individuals, positioning it in 3rd place of most common cancers worldwide in 2020 (1). Although more effective treatment procedures for CRC have been developed in recent years, the number of patients not responding to chemotherapy is still relevant (2). Besides, the patient relapse and metastasis have been associated with cell subpopulations present in solid tumors called tumor-initiating cells (TICs) or cancer stem cells (CSCs) (3-6).

CSCs share properties with mesenchymal stem cells (MSCs) such as self-renewal and differentiation. Specifically, CRC-CSCs present several surface markers such as CD144, CD166, CD29, ALDH1, LGR5 and CXCR4 but mainly CD133, CD24, and CD44 (7,8). Moreover, SOX2, OCT4, NANOG, and KLF4 transcription factors (TFs) are predominantly expressed in CSCs with a pivotal function in pluripotency maintenance (9-11).

Recently, CSCs and epithelial-mesenchymal transition (EMT) phenotype has been associated with the ability to invade and metastasize through the trans-differentiation of epithelial cells into a mesenchymal state (12,13). This event is mainly regulated by the activation of several genes, which turn off E-cadherin (*CDH1*) and turn on N-cadherin (*CDH2*) and Vimentin (*VIM*) (14,15). Also, this EMT activation is regulated by β-catenin (*CTNNB1*), the primary molecular effector of the Wnt signaling pathway (16).

Another essential regulator of the EMT phenotype is the SRY-box transcription factor 9 (*SOX9*), which also regulates organogenesis (17) and cell differentiation (18,19) in several tissues. SOX9 has a key role in the development of human cancer (20). Specifically, SOX9 has been associated to tumor progression, advanced tumor stage, and lower overall survival in CRC patients (21-24). While this participation of SOX9 on CRC has been demonstrated, its relationship with the stem phenotype has not been yet elucidated. Thus, we aim to assess the role of SOX9 in CRC-CSCs phenotype maintenance. We present this article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1163/rc).

Highlight box

Key findings

- SOX9's absence affects the epithelial cell morphology and the pluripotency signature of the undifferentiated colorectal cell line HCT116.

What is known and what is new?

- SOX9 is an important transcription factor involved in the maintenance of stemness properties of normal colon cells.
- This is the first report showing evidence of SOX9 participation in CRC cell morphology. Also, we suggest two independent feedback loop effects between SOX9 and SOX2 as well as CD73.

What is the implication, and what should change now?

- More experiments are needed to deepen the feedback loop processes that we propose here, as well as the participation of SOX9 in cell morphology.

Methods

Cell culture

The human CCR cell lines Caco-2, HT-29, HCT116 y SW-480 as well as the normal colon cell line CCD18-co, were obtained from American Type Culture Collection (ATCC). Culture conditions are those used by Lizárraga-Verdugo *et al.* (25). This study was conducted under the principles of the Declaration of Helsinki (as revised in 2013).

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA isolation and conditions for real-time PCR were developed following the methods of Lizárraga-Verdugo *et al.* (25). Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, CA, USA) and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, MA, USA) for *SOX9* (Hs00165814_m1), *OCT4* (Hs04260367_gH), *KLF4* (Hs00358836_m1), *SOX2* (Hs04234836_s1), *NANOG* (Hs02387400_g1), *CTNNB1* (Hs00355045_m1), *VIM* (Hs00958111_m1), *CDH1* (Hs01023895_m1), *CDH2* (Hs00983056_m1) and *ACTB*

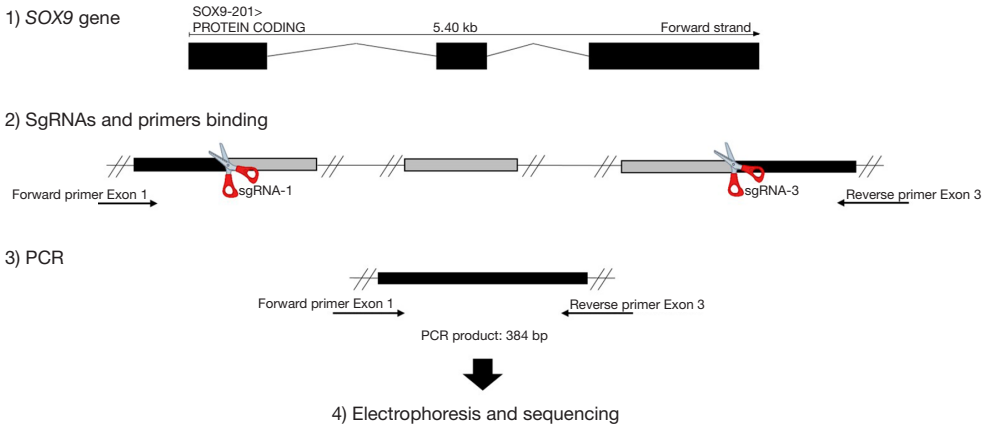

Figure 1 Methodology strategy applied to *SOX9-KO* cells generation by CRISPR-Cas9 system in HCT116 line. Targeting strategy for the KO of the *SOX9* gene, showing the sgRNAs and the primers designed to specifically bind to exons 1 and 3 to perform KO and detection by PCR, respectively, to finally sequence the resulting PCR product. SOX9, SRY-box transcription factor 9; PCR, polymerase chain reaction; KO, knockout; sgRNAs, single guide RNAs.

(Hs01060665_g1) as endogenous control.

Flow cytometry immunophenotyping

Cultured cells were washed with phosphate-buffered saline (PBS) and resuspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) at a density of 1×10^5 cells/100 µL. Single cells were stained using antibodies against CSCs; anti-human CD133-APC (Miltenyi Biotec, NRW, Germany), anti-human CD44-FITC (Miltenyi Biotec), and anti-human CD24-FITC (Miltenyi Biotec), and the Human MSC Analysis Kit (BD Biosciences, NJ, USA); CD90-FITC, CD105-PerCP-Cy5.5, and CD73-APC. Cells were incubated with the antibodies for 15 min at room temperature, washed with PBS, and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences) and the software FlowJo v.10.

SOX9 knockout (KO) by CRISPR-Cas9

SOX9 KO was achieved using the CRISPR-Cas9 system. Single guide RNAs (sgRNA) for exon 1 and 3 of the *SOX9* gene were designed using the Web application https://benchling.com/, obtaining the gene-specific sgRNA sequences 5'-CAGGAGAACACGTTCCCCAA-3' for exon 1 and 5'-ACGTCGCGGAAGTCGATAGG-3' for exon 3 (both synthesized by Integrated DNA Technologies, IDT, IA, USA). Cells (3×10^5) were grown on 6-well plates to perform a transfection of the CRISPR-cas9 system as follows: 26.9 µL Opti-MEM (Gibco, CA, USA), 2.6 µL Cas9 Plus Reagent (Invitrogen, CA, USA), 6,250 ng TrueCut Cas9 Protein v.2 (Invitrogen) and 100 pg of each sgRNAs, at the same time Lipofectamine CRISPRMAX Cas9 (Invitrogen) were diluted in 29.3 µL Opti-MEM. Both mixtures were incubated separately for 5 min at room temperature, then mixed and added to the cells. The cells were incubated with the mixture for 48 h and subsequently harvested to proceed with the cloning of single cells in a 96-well plate; when reaching sufficient cell density, a part of the cloned cells was taken for evaluation by PCR using the following primers for exon 1: forward 5'-CCCGCGTATGAATCTCCTG-3', and the reverse for exon 3 5'-TGCTTGGACATCCACACG-3' (both synthesized by IDT). This generated 384 bp PCR product was resolved by electrophoresis in 1.5% agarose gel at 100 V and analyzed by Sanger sequencing (Eton Bioscience, Inc., CA, USA) to confirm the *SOX9-KO* (*Figure 1*).

Immunofluorescence

For SOX9 localization 8×10^4 cells were grown in round-glass coverslips into a 24-well plate. Conditions and concentrations are those used by Lizárraga-Verdugo *et al.* (25). Finally, actin filaments were stained with 1:2,000 Phalloidin-iFluor 488 Reagent (Abcam, ab176753, Cambridge, UK) for 5 min, washed, and mounted with Fluoroshield with DAPI (Sigma-Aldrich, MA, USA). Visualization was obtained at a 40× magnification in a

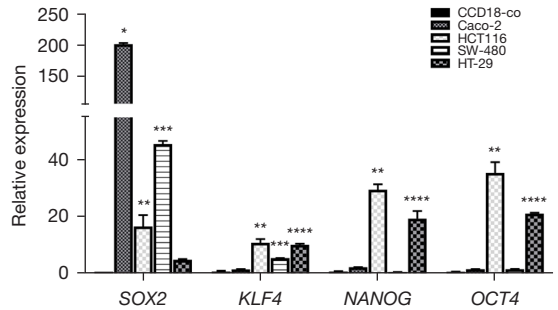

Figure 2 Colorectal cancer cell line HCT116 has the strongest stem phenotype. HCT116 overexpressed most transcription factors related with CSCs as compared to Caco-2, SW-480, HT-29 and the non-tumorigenic colon cell line CCD18-co. P<0.05 for Caco-2 (*), HCT116 (**), SW-480 (***) or HT-29 (****) *vs.* CCD18-co.

Leica LSCM (TCS SP8, Leica Microsystems, Wetzlar, Germany) and F-actin quantification was performed by phalloidin analysis of the image using the Leica software LAS X (v.3.5.6.21594) to obtain the mean values of relative fluorescence. Analyses were performed in triplicates from three different regions of interest (ROIs).

Sphere-formation, self-renewal, and differentiation assays

Cells were detached with 0.05% trypsin/EDTA (Gibco), and the resulting dissociated single cells were diluted to a density of 1×10^4 cells and placed into ultra-low attachment (ULA) 24-well plates with sphere formation medium (SFM), composed of serum-free medium DMEM/F12 supplemented with 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF) (both from ABM, BC, Canada) and 1× B27 supplement (Gibco). The cells were incubated at 37 ℃ with 5% CO_2 for 14 days to let the formation of first-generation colonospheres.

To stimulate no CSCs differentiation or to evaluate self-renewal, first-generation spheroids were harvested and enzymatically dissociated with ACCUTASE (STEMCELL technologies, WA, USA) and gentle pipetting, then, 1×10^4 single cells, were re-plated and incubated again for 14 days, either in SFM, to examine the ability of colonospheres to form second-generation spheroids, or in DMEM/F12 supplemented with 15% FBS, to evaluate the loss of stemness maintaining ULA conditions (differentiation) by CD44, CD133 and CD24 evaluation using flow cytometer (previously mentioned). The number of spheres was assessed by counting under an inverted microscope using 10× objective. Also, for cell density analysis we performed measurements by using ImageJ software (https://imagej.nih.gov/ij/).

Statistical analysis

Three independent experiments in triplicate were performed and results were calculated as means ± standard deviation (SD) and analyzed using GraphPad Prism 8 software (MA, USA). Statistically significant differences between groups were assayed by a two-tailed *t*-test; multiple comparisons were tested by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. A P<0.05 was considered significant.

Results

HCT116 exhibits more stemness phenotype than other CRC cell lines

To determine the colorectal cell line with more stem phenotype, we analyzed the expression of the pluripotency genes *SOX2*, *KLF4*, *NANOG*, and *OCT4* by RT-qPCR in the CRC cell lines HCT116, HT-29, Caco-2, SW480 and the normal colon cell line CCD18-co. Caco-2 showed the highest levels of *SOX2* in comparison to the control [fold change (FC =202.0, P<0.0001]; however, HCT116 was the cell line with the highest level of *KLF4* (FC =11.1, P<0.001), *NANOG* (FC =29.6, P<0.001) and *OCT4* (FC =35.5, P<0.001), but the third in expression of *SOX2* (FC =16.8, P<0.0001) (*Figure 2*). To help guide our final selection, we also analyzed the surface markers CD44, CD133, and CD24 related to CRC-CSCs. HT-29 cells were almost all $CD24^+$ cells (99.7%±0.1%), followed by SW-480, Caco-2 and HCT116, which showed more moderate positive levels (69.0%±16.1%, 67.1%±5.9% and 47.2%±2.06%, respectively). HCT116 had the highest levels of $CD44^+$ cells (98.7%±1.0%) and the second-high population of $CD133^+$ (90.9%±2.9%). Considering this, we settled for HCT116 for its high stem phenotype (*Table 1*).

Absence of SOX9 induced morphological changes in HCT116

To assess the role of *SOX9* gene in the stemness properties of the CRC HCT116 cell line, we performed a *KO* using the CRISPR-Cas9 system, obtaining one modified clone in which SOX9 protein expression was abolished. Our *SOX9-KO* clone had a deletion of 2,101 bp between exon 1 and 3, in both alleles (*Figure 3A*,*3B*). We then compared

the expression of SOX9 at the protein (*Figure 4A,4B*) and mRNA (FC =0.01, P=0.0237) (*Figure 4C*) levels in both, the KO and parental cell lines, confirming that SOX9 was absent in *SOX9-KO*. Also, phalloidin levels were quantified showing a significant inhibition of this in *SOX9-KO* cells (P=0.0001) (*Figure 4B*). In addition, morphology analysis using Wright's stain showed strikingly different morphology between *SOX9-KO* and its parental cell line; while parental HCT116 cells showed an expanded cytoplasm with a flatted, enlarged, and spread morphology, *SOX9-KO* cells displayed less cytoplasm with a spindle-shaped fibroblast-like morphology (*Figure 4D*). Despite the need for further studies, this morphological change could be explained by the change in actin filament reorganization, as observed by phalloidin stain (*Figure 4A*). These results suggest that SOX9 plays a role in the epithelial morphology of the HCT116 cell line.

Table 1 Proportion of positive cells for CD133, CD44, and CD24 in colorectal cancer cell lines HCT116, HT-29, Caco-2, SW-480 and in normal colon cell line CCD18-co

Cell line	% CD133	% CD44	% CD24
HCT116	90.9±2.9	98.7±1.0	47.2±2.06
HT-29	42.4±10.0	66.8±11.5	99.7±0.1
Caco-2	97.8±1.1	14.3±2.7	67.1±5.9
SW-480	0.4±0.2	81.6±8.1	69.0±16.1
CCD18-co	13.1±0.9	98.5±1.8	96.9±0.8

KO of SOX9 in HCT116 affected its pluripotency signature, stem-cell markers, and EMT phenotype

We evaluated the pluripotency genes *SOX2*, *KLF4*, *NANOG*, and *OCT4* by RT-qPCR to elucidate the effect of *SOX9* deletion on stemness properties, finding a significant overexpression of *SOX2* (FC =2.5, P<0.0001) and *KLF4* (FC =1.9, P<0.0001) in *SOX9-KO* in comparison with parental cell line HCT116, and a minimal non-significant rise of *OCT4* (FC =1.2, P=0.0983) and *NANOG* downregulation (FC =0.7, P=0.0064) (*Figure 5A*). This indicates that *SOX9* participates in the pluripotency signature. To expand our stem phenotype characterization, we measured MSCs surface markers such as CD90, CD105, and CD73 in both *SOX9-KO* and parental HCT116 line, finding a very low proportion of CD90 (0.9%±0.8% *vs.* 0.2%±0.2%, P=0.510), and CD105 (2.8%±0.5% *vs.* 0.7%±0.3%, P=0.052) positive cells; however, we found a significant inhibition of CD73 (43.3%±3.9% *vs.* 95.2%±2.9%, P=0.002), meaning that the expression of this marker could be affected by SOX9 absence. To evaluate if *SOX9* absence affects EMT, we analyzed *VIM*, *CDH1*, *CDH2*, and *CTNNB1* expression by RT-qPCR, finding a downregulation of *VIM* (FC =0.2, P=0.0017) in *SOX9-KO* cells in comparison with parental cells; in contrast, we observed an upregulation of *CDH2* (FC =1.48, P=0.0363) (*Figure 5B*) in *SOX9-KO* cells. This suggests that the lack of *SOX9* has a minimal impact on the EMT phenotype.

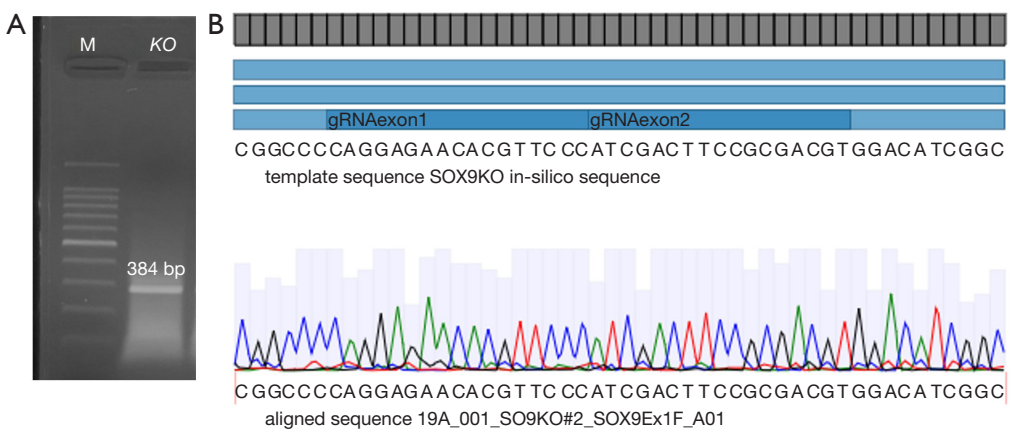

Figure 3 *SOX9-KO* cells generation by CRISPR-Cas9 system in HCT116 line. (A) Gel electrophoresis shows the partial loss of *SOX9* gene with the amplification of a PCR product of 384 bp, employing primers guided to EXON1 and 3. (B) Confirmatory sequencing of the PCR product that exhibits the site of binding of the single guide RNA-1 and -3 with the loss of ~2,100 bp in the *SOX9* gene. M, molecular markers; KO, knockout; SOX9, SRY-box transcription factor 9; PCR, polymerase chain reaction.

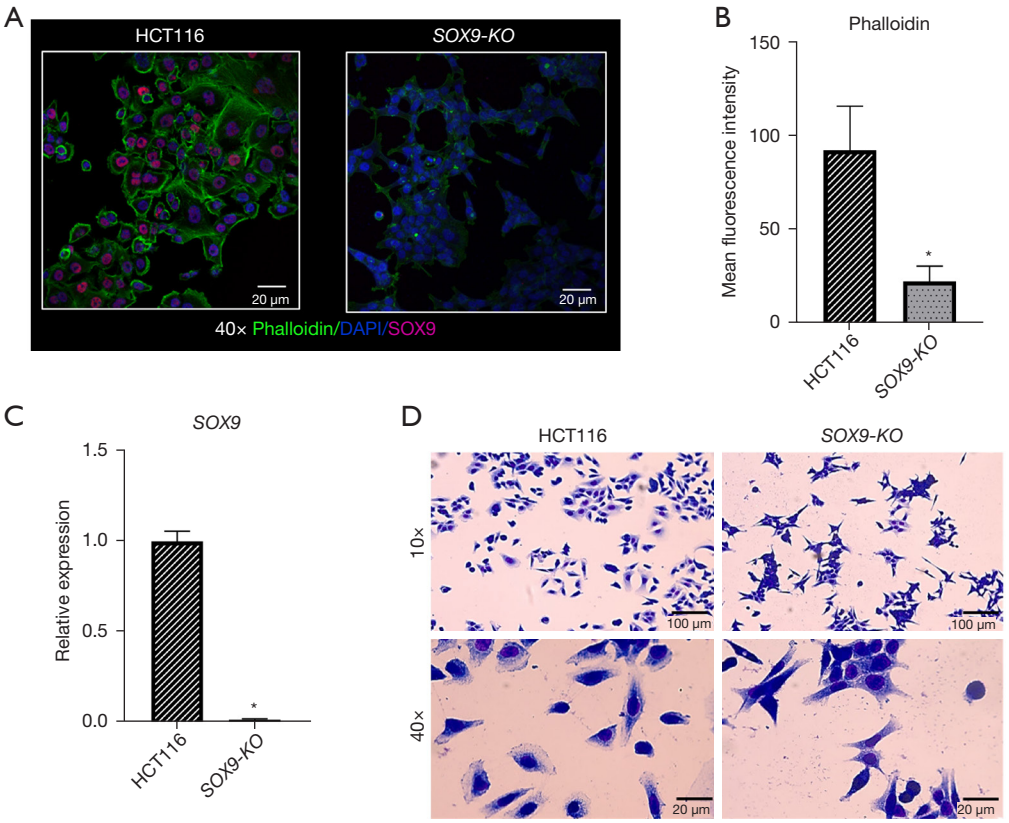

Figure 4 A *SOX9-KO* clone with modified morphology was generated by CRISPR-Cas9 system in the HCT116 line. (A) Immunofluorescence staining with phalloidin, DAPI and anti-*SOX9*, shows the SOX9 absence in *SOX9-KO* at a magnification of 40×; (B) F-actin quantification by phalloidin staining in *SOX9-KO* cell *vs.* control; (C) relative expression of *SOX9* mRNA in *SOX9-KO* in comparison with its parental cell line; and (D) morphology analysis by Wright's staining in *SOX9-KO* and parental line at 10× and 40× magnifications exhibits a change of the cell morphology from epithelial- to fibroblast-like and actin filaments reorganization. *, P<0.05. SOX9, SRY-box transcription factor 9; KO, knockout.

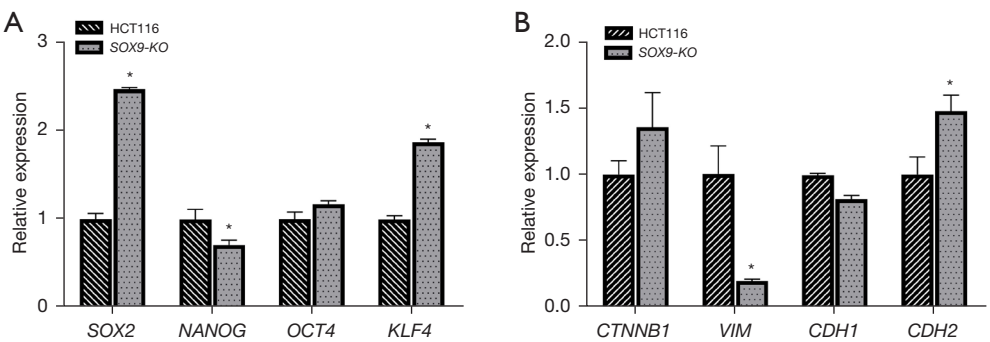

Figure 5 Knocking down *SOX9* in HCT116 line affects its expression of pluripotency signature and maintains EMT phenotype. Relative expression of (A) *SOX2*, *NANOG*, *OCT4* and *KLF4*; genes related to CSCs; and (B) *CTNNB1*, *VIM*, *CDH1* and *CDH2*; EMT-related genes in *SOX9-KO* and its parental line. *, P<0.05. SOX9, SRY-box transcription factor 9; KO, knockout; EMT, epithelial mesenchymal transition; CSC, cancer stem cell.

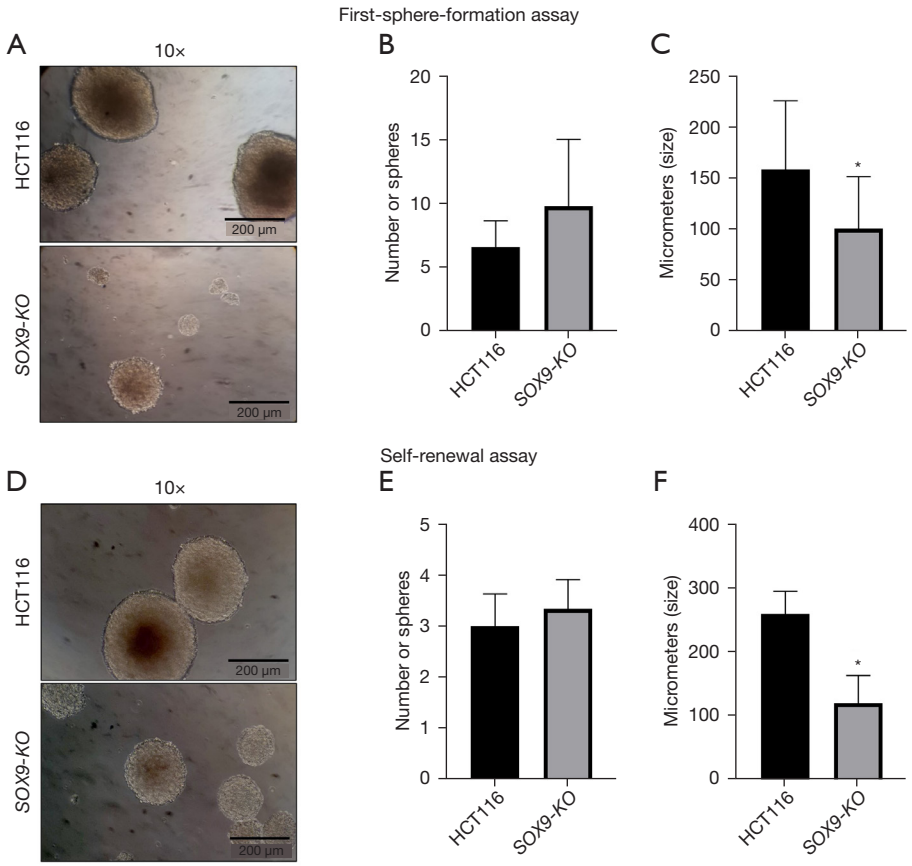

Figure 6 *SOX9-KO* affects first- and second-sphere formation. (A) Sphere-formation assay performed in ULA conditions with the SFM in *SOX9-KO* and its parental line, showing less densities and weaker spheres in the KO at a magnification of 10×; (B) not significant changes were found in the number of spheres between *SOX9-KO* cells *vs.* control; and (C) a significant larger size in spheres derived from the parental cells *vs. SOX9-KO* cell was found. (D) Self-renewal capability exhibits similar morphology characteristics; and (E) quantity as the first generation; however, (F) the effect on the size of the spheres was maintained during the second generation derived from *SOX9-KO*. *, P<0.05. SOX9, SRY-box transcription factor 9; KO, knockout; ULA, ultra-low attachment; SFM, sphere formation medium.

The absence of SOX9 inhibits tumorigenesis during the first- and second-sphere formation

To evaluate the effect of *SOX9* deletion on tumorigenesis of CRC HCT116 line, we analyzed colonospheres generated in our first sphere-formation assay. The shape of the HCT116-spheres was spherical with regular and continuous contour, which can be described as compact tumor-packaging, meanwhile, *SOX9-KO*-spheres formed structures composed of rounded cells with weak neighboring cell-cell interactions (*Figure 6A*). *SOX9-KO* cells had a slightly higher tendency [P value = not significant (ns)] to form more spheres of their weaker and less compact characteristics (*Figure 6B*). Also, parental HCT116 cells formed larger spheres with diameters varying from 70 to 230 µm (*Figure 6C*), in comparison to *SOX9-KO*-spheroids which had a significantly lower cell density and size (40–160 µm) (P=0.0009) (*Figure 6C*).

Next, we evaluated their self-renewal capability (second-colonospheres generation) finding similar observations to those of the first-sphere generation assay, in both the parental and *SOX9-KO* spheres (*Figure 6D-6F*). The above results suggest that *SOX9-KO* cells are inefficient in maintaining stable spheroids and triggering more spheres formation with less size. We hypothesize that this phenomenon is linked to morphological changes and its possible structural cytoskeleton behavior after *SOX9* abolition.

Table 2 Percentage of positive populations to CSCs surfaces markers CD133, CD44, and CD24 in *SOX9-KO* and its parental cell line HCT116, before and after the first- and second-colonosphere formation and differentiation assays

Cell types	% CD133	% CD44	% CD24
Basal			
HCT116	90.9±2.9	98.7±1.0	47.2±2.06
SOX9-KO	87.8±3.1	61.8±14.4[a]	45.1±10.3
First-generation-spheres			
HCT116	90.5±1.3	96.0±6.0	67.1±11.9
SOX9-KO	79.7±5.4	88.0±12.4	75.0±12.2[b]
Second-generation-spheres			
HCT116	92.8±1.7	98.7±1.01	79.4±8.9
SOX9-KO	88.8±2.7	97.3±0.9	83.6±18.7
Differentiation assay			
HCT116	11.9±0.0[c]	82.8±0.2	24.8±2.4[c]
SOX9-KO	17.2±0.0[c]	71.3±3.8[c]	22.8±3.4[c]

[a], P<0.05 comparing HCT116 *vs.* *SOX9-KO* basal surface marker proportions; [b], P<0.05 comparing HCT116 and *SOX9-KO* from first-generation-spheres *vs.* basal marker proportions; [c], P<0.05 comparing HCT116 and *SOX9-KO* from differentiation *vs.* first-generation-spheres marker proportions. The data are shown as mean ± standard deviation. CSC, cancer stem cell; SOX9, SRY-box transcription factor 9; KO, knockout.

SOX9 KO does not affect CSCs enrichment during sphere-formation assays

To evaluate the effect of *SOX9* deletion on CSCs surface markers before and after first- and second-colonospheres formations, we analyzed the surface expression of CD133, CD44, and CD24 markers in parental and KO lines. During conventional cell culture (before spheres formation) we observed a significant inhibition of the initial basal levels on the positive population to CD44 from 98.7% in parental cells to 61.8% in *SOX9-KO* (P=0.042). In contrast, the population positivity for CD24 and CD133 did not change their proportions, showing ~90% and ~45% of cell positivity in both cell lines (*Table 2*).

During the first-colonosphere generation assay, we found similar proportions of CD44 and CD24 in both groups, while CD133 population was lower (90.5%, P=0.052) in *SOX9-KO* (79.7%) as compared to HCT116 (*Table 2*). Similarly, after the sphere self-renewal assay (second-colonosphere formation) no changes were found between *SOX9-KO* and HCT116 in any marker proportion (*Table 2*).

When comparing CSCs marker levels of first-generation-colonospheres, in KO and parental cells, against their respective initial basal levels, *SOX9-KO* line showed no significant changes for CD133 (from 87.8% to 79.7%, P=0.240) and CD44 (from 61.8% to 88.0%, P=0.220), and a significant increase on CD24 from 45.1% to 75.0% (P=0.015). No changes were found for HCT116 in any marker (*Table 2*). Additionally, a comparison between first- and second-generation-colonospheres, showed no differences in any marker for both lines.

SOX9 does not associate the loss of CSCS markers, in both the KO and its parental line

To investigate the role of SOX9 on CSCs differentiation, we promoted differentiation to no CSCs of dissociated first-colonospheres by adding 15% FBS and keeping ULA conditions. Differentiated cultures were then analyzed for CSCs differentiation surface markers. Both, HCT116 and *SOX9-KO* cells diminished their percentages of CD133 (90.5%±1.3% *vs.* 11.9%±0.0%, P=0.0001; 79.7%±5.4% *vs.* 17.2%±0.0%, P=0.0002, respectively) and CD24 (67.1%±11.9% *vs.* 24.8%±2.4%, P=0.023; 75.0%±12.2% *vs.* 22.8%±3.4%, P=0.010, respectively) positive cells (*Table 2*). Considering that *SOX9-KO* cells observed the same behavior as its parental line, with a fall on CD44 (88.0%±12.4% *vs.* 71.3%±3.8%, P=0.006) positive cells (*Table 2*), we concluded that *SOX9* does not seem to participate in the loss of HCT116 CSCs surface markers.

Discussion

SOX9 is often overexpressed in solid cancers, including CRC (26), but its role in CSCs maintenance is unclear. In this work, we reported a clear association between *SOX9* and CSCs characteristics in a CRC line.

The co-expression of *SOX2*, *OCT4*, *KLF4*, and *NANOG* is essential to promote stemness by upregulating genes involved in self-renewal and pluripotency and suppressing genes implicated in cell differentiation (27). In this regard, we measured the expression of these TFs in CRC cell lines, our finding showed that HCT116 exhibits the strongest stem phenotype of the cell lines analyzed (*Figure 2*). In agreement with our results (*Table 1*), it has been reported that HCT116 exhibits a low $CD24^+$ accompanied by a moderate (28) or high CD44/CD133 population (29).

Previously, our research group found that SOX9 is overexpressed in HCT116 compared to other CRC cell lines (26) and was, therefore, the cell line of choice to KO the *SOX9* gene (*SOX9-KO*).

As an effect, we observed that cell morphology, actin-filaments reorganization, and f-actin were seriously affected in *SOX9-KO* (*Figure 4A*,*4D*). A previous study showed that ectopic SOX9 expression turns on a flatter morphological cell appearance as compared with the control in prostate cancer cell lines; the above is due to overexpression of genes involved in cell adhesion and shape (30).

An increased expression of *SOX2* and *KLF4* and decreased *NANOG* were observed in our *SOX9-KO* cells (*Figure 5A*). Interestingly; the reduction of *SOX9* expression resulted in a feedback loop effect with *SOX2*, due to their reciprocal binding to their promoter regions in breast cancer cell lines (31). While the above findings derived from a partial silencing of *SOX9*, our findings resulted from a KO, suggesting a possible compensatory process due to a total absence of *SOX9* and resulting in a *SOX2* overexpression. In addition, ectopic *SOX9* expression downregulates *KLF4* expression (32). Interestingly, in normal colon, *KLF4* is highly expressed in differentiated cells (33) and poorly expressed in the crypts where the undifferentiated cells are found; meanwhile, *SOX9* has the opposite pattern (33). Together, these results support the regulatory role of *SOX9* on pluripotency genes related to the stemness phenotype of the undifferentiated CRC cell line HCT116.

In the MSCs markers context, reports have shown that CD73 promotes *SOX9* transcription, and protects to *SOX9* from degradation in hepatocellular carcinoma (34). Interestingly, as the sudden decrease in $CD73^+$ cells in our *SOX9-KO* we propose a possible positive regulation of CD73 by *SOX9*. However, more information is needed to understand this mechanism.

SOX9 is an important promoter of EMT phenotype (35), however, we observed a maintenance of this phenotype, even with the absence of *SOX9*. Interestingly, CD73 has been related to EMT since it regulates at transcriptional levels essential EMT driver genes such as *TWIST1*, *SNAI1*, and *ZEB1* (36). As we showed before, we have a partial presence of this marker, showing that the EMT phenotype could be gained in *SOX9* absence. Further experiments must confirm the above to elucidate their specific role.

Interestingly, we obtained a tendency to a larger number of small size spheres in the *SOX9-KO* cell line, in both first- and second generation-spheres (*Figure 6*), the above possibly due to by the morphology change present by *SOX9* abolition which result in labile spheres.

We found an initial effect on CD44 basal levels in *SOX9-KO* line (*Table 2*), but only before the first and second sphere-formation assays; these results could be due to the effect of components of the sphere medium such as the B27, which allows tumor-spheres growth in the absence of FBS, promoting CSCs enrichment (37); also, the presence of FGF and EGF factors which promote CSCs enrichment (38-40). Interestingly, we found an increase of CD24 after first-generation-spheres of *SOX9-KO* cells, in comparison with its own initial basal level.

Finally, it has been demonstrated that the presence of FBS stimulates the loss of the surface markers related to CSCs; such as CD24, which declined in CSCs derived from hepatocellular carcinoma tissues, promoting the differentiation and recovering the phenotype of the cells before CSCs enrichment (41). This suggests that FBS stimulates the loss of CSCs by cell differentiation, which was observed in our results. We must highlight here the need of our research field to harmonize culture conditions for cell lines, amenable with proper *in vivo* cell micro-environments. The use of xenogeneic factors or the unsuspected presence of endotoxins in our culture media must be carefully considered giving preference to human additives rather than bovine.

Conclusions

In conclusion, *SOX9* absence affects the epithelial morphology and the pluripotency signature of the HCT116, an undifferentiated colorectal cell line. We found an increase in *SOX2* expression as a compensatory mechanism by SOX9 lack, and the increase of *KLF4* could be associated with an early differentiation process. Also, *SOX9* is associated with the presence of CD44 and CD73 positive populations, being the last one a putative feedback loop effect between CD73 and *SOX9*. Interestingly, the EMT phenotype is still occurring in *SOX9-KO* cells, possibly by compensatory mechanisms. The lower size and compaction of the spheres in *SOX9-KO* could be influenced by the morphology change. Collectively, these results provide evidence that *SOX9* promotes the maintenance of stemness properties in CRC-CSCs.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1163/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-1163/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted under the principles of the Declaration of Helsinki (as revised in 2013).

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