Peer Review File

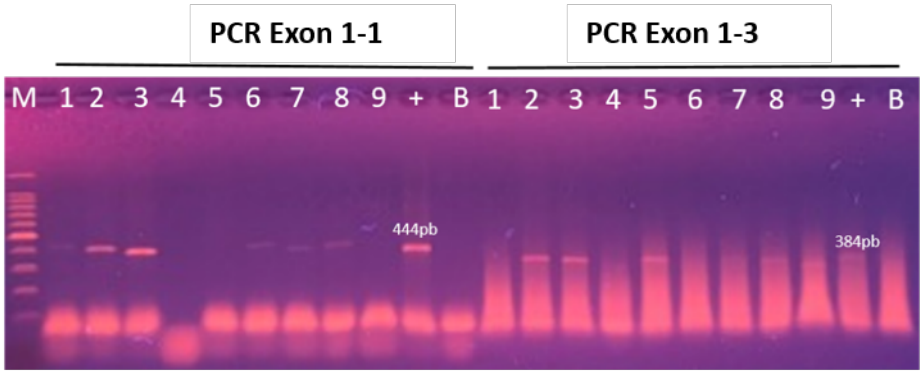
Article Information: https://dx.doi.org/10.21037/jgo-22-1163

Reviewer A

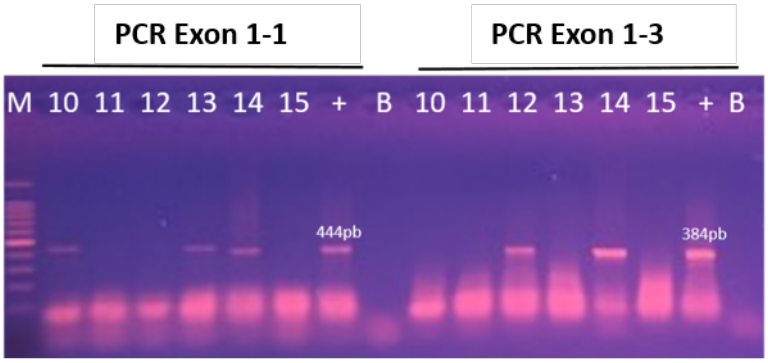
In this manuscript entitled "SOX9 knockout decreases stemness properties in colorectal cancer cells", Avendano-Felix investigates the consequences of SOX9 ablation on a colorectal cancer cell line.
Modulation of stem cell associated marker expression and changes in the cellular phenotype are observed upon deletion of the gene, suggesting a role for SOX9 for cancer stem cell properties. However, some of the conclusions presented here are not supported by the data presented. Importantly the authors need to report how many different mutated clones were assessed to ensure reproducibility of the phenotype.

Detailed comments:
It is necessary to include a figure showing the gene deletion strategy and the characterisation of the resulting deleted allele analysed by PCR, as described in the material and methods as data not shown. This is an essential requirement to assess success of the gene deletion. How many mutant clones were obtained? How many independent clones were analysed in this study to assess reproducibility of the phenotype? Crispr/Cas gene editing technology is known to be associated with off target effects; it is thus necessary to ensure that several independent clones show the same phenotype to ensure that the alterations observed are solely due to deletion of SOX9.

We kindly accept your suggestion; we modified material and methods section adding *KO* strategy as Figure 2A (See page 7, line 132, since here the sequence of the figures was modified). Also, we added confirmatory PCR product and the result of the sequencing in Figure 2B and C (See page 9, line 188)
We obtained several mutated clones from HCT116 knocked out using CRISPR/Cas9 with both, one allele and double allele SOX9 gene abolition. Here, we kindly provide a figure of a PCR electrophoresis profile showing different clones obtained from same gene editing procedure.
Unfortunately, we only used only one mutated clone to achieve experiments (this was already specified in page 9, line 186) due to these experiments were performed during pandemic times and laboratory's activities were restricted in our institution, in that way, other clones obtained were preserved for future experiments. We attached below examples of the identification of 3 KO clones by PCR.

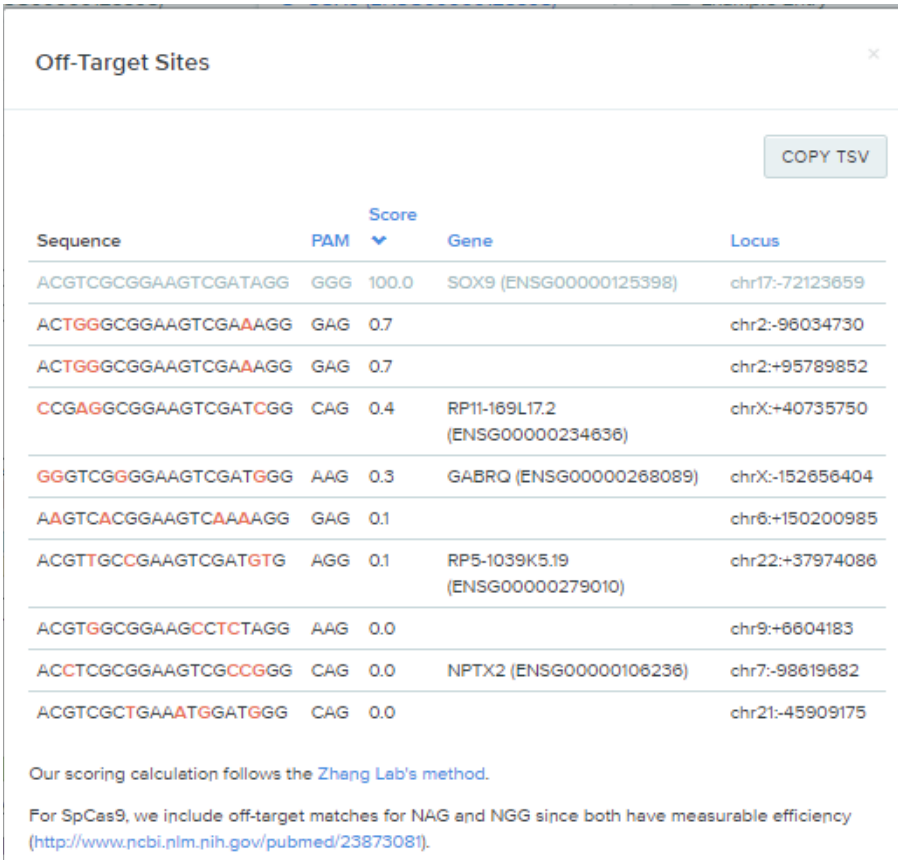


	1	2	3	4	5	6	7	8	9
Exon 1-1	+	+	+	-	-	+	+	+	-
Exon 1-3	-	+	+	-	+	-	+	+	+
INDEL	WT	+/-	+/-	-	KO	WT	+/-	+/-	KO



	10	11	12	13	14	15
Exon 1-1	+	-	-	+	+	-
Exon 1-3	-	-	+	-	+	-
INDEL	WT	-	KO	WT	+/-	-

We used Brenchling software and prediction showed that both guide RNAs used in our experiments were only complementary to SOX9 (100 %). We attached below the predictions results with no potential off-targets for both guide RNAs (All below 1% possible complementarity).



Off-Target Sites

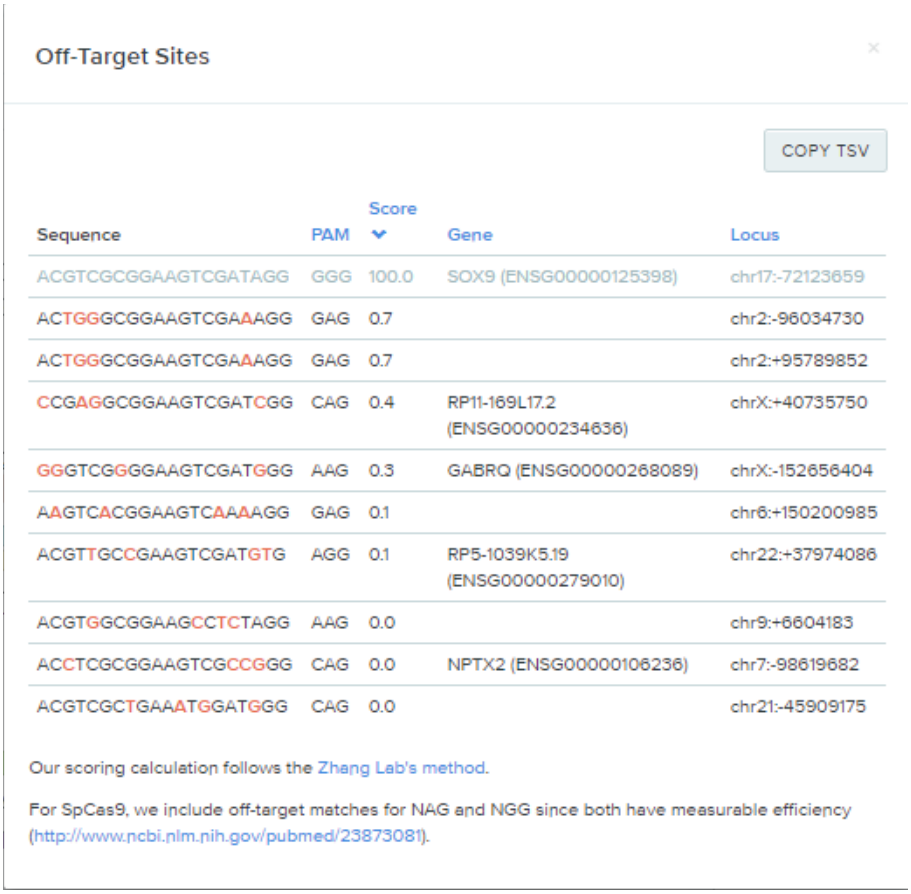
COPY TSV

Sequence	PAM	Score	Gene	Locus
ACGTCGCGGAAGTCGATAGG	GGG	100.0	SOX9 (ENSG00000125398)	chr17:-72123659
ACTGGGCGGAAGTCGAAAGG	GAG	0.7		chr2:-96034730
ACTGGGCGGAAGTCGAAAGG	GAG	0.7		chr2:+95789852
CCGAGGCGGAAGTCGATCGG	CAG	0.4	RP11-169L17.2 (ENSG00000234636)	chrX:+40735750
GGGTCGGGGAAGTCGATGGG	AAG	0.3	GABRQ (ENSG00000268089)	chrX:-152656404
AAGTCACGGAAGTCAAAAGG	GAG	0.1		chr6:+150200985
ACGTTGCCGAAGTCGATGTG	AGG	0.1	RP5-1039K5.19 (ENSG00000279010)	chr22:+37974086
ACGTGGCGGAAGCCTCTAGG	AAG	0.0		chr9:+6604183
ACCTCGCGGAAGTCGCCGGG	CAG	0.0	NPTX2 (ENSG00000106236)	chr7:-98619682
ACGTCGCTGAAATGGATGGG	CAG	0.0		chr21:-45909175

Our scoring calculation follows the Zhang Lab's method.

For SpCas9, we include off-target matches for NAG and NGG since both have measurable efficiency (http://www.ncbi.nlm.nih.gov/pubmed/23873081).

Figure with off-target predictions of the guide RNA for the exon 1 employed in our CRISPR/Cas9 procedure.



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GGGTCGGGGAAGTCGATGGG	AAG	0.3	GABRQ (ENSG00000268089)	chrX:-152656404
AAGTCACGGAAGTCAAAAGG	GAG	0.1		chr6:+150200985
ACGTTGCCGAAGTCGATGTG	AGG	0.1	RP5-1039K5.19 (ENSG00000279010)	chr22:+37974086
ACGTGGCGGAAGCCTCTAGG	AAG	0.0		chr9:+6604183
ACCTCGCGGAAGTCGCCGGG	CAG	0.0	NPTX2 (ENSG00000106236)	chr7:-98619682
ACGTCGCTGAAATGGATGGG	CAG	0.0		chr21:-45909175

Our scoring calculation follows the Zhang Lab's method.

For SpCas9, we include off-target matches for NAG and NGG since both have measurable efficiency (http://www.ncbi.nlm.nih.gov/pubmed/23873081).

Figure with off-target predictions of the guide RNA for the exon 3 employed in our CRISPR/Cas9 procedure.

Also we evaluated phalloidin in another mutated clones showing the same change of cell morphology exhibited by the clone employed in this research (Below we attached an example of another KO clone resulted by the same gene editing).

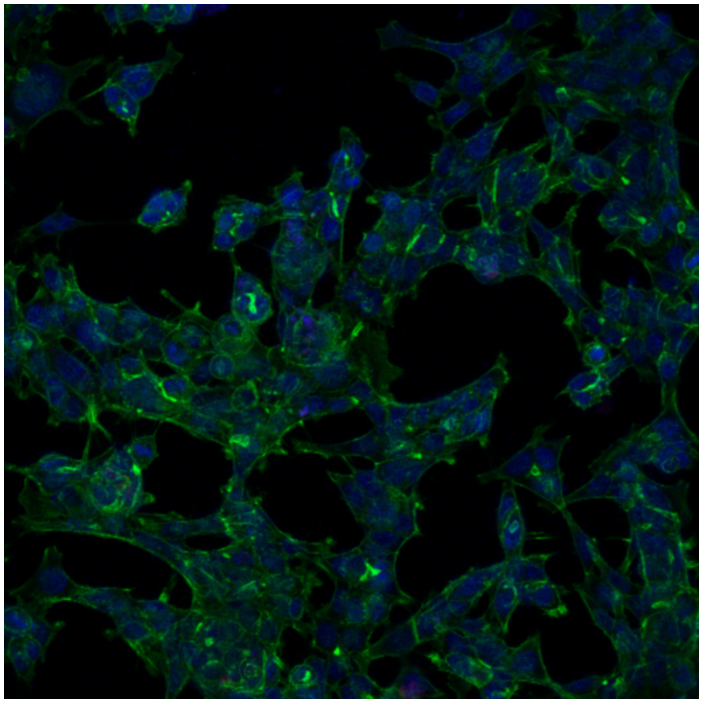


Figure2: there is no assessment/quantification for the altered cell morphology presented, either phalloidin levels or cell morphology while in the discussion this is reported as a "seriously affected". Despite their repeated claims, the author fail to provide a significant assessment of the loss of epithelial properties upon SOX9 deletion. This phenotype must be better assessed and quantified.

We analyzed morphological changes by using Wrigth's stain in parental HCT116 compared to SOX9 KO culture too. The use of phalloidin stain was used in order to show morphological differences in those SOX9 absent cell lines culture., however we already added a quantification of phalloidin levels (See page 7, line 142-146 and page 9, line 190-191).

In figure 3, changes in CDH1 and CTNNB1 expression levels are not significant and thus should not be presented in the text as being respectively down and up-regulated. Their expression is not significantly affected thus nothing can be concluded from these results.

We already attended this suggestion (We modified page 10, line 212).

Figure 4D-F, the size of SOX9KO spheres is described as tending to be larger, but without statistical significance (line 221 and 554-555), while the data is actually showing the opposite (Fig.4F, SOX9KO are significantly smaller). Do the authors mean that the secondary SOX9KO spheres appear larger than the primary SOX9KO ones? This should be made clearer in the text and figure legend.

We appreciate your comments and we checked the text and made edition of the text that we

believe can make a difference in the understanding about the quantity of spheres among control and KO cells (See page 10, line 222-226 and page 17, line 386-391).

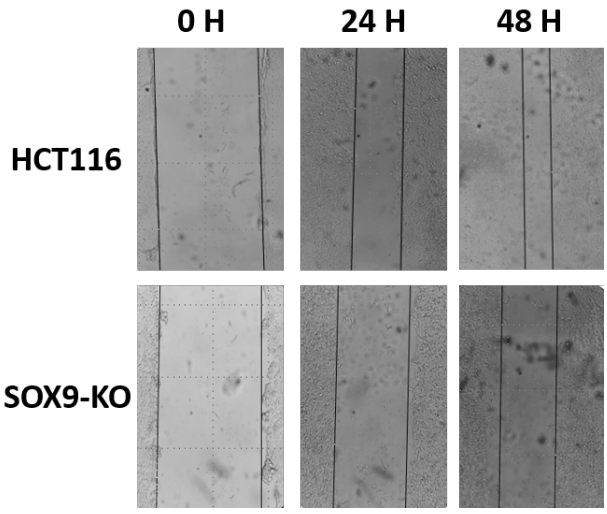
SOX2 and SOX9 belong to different subgroups of the SOX gene family, respectively B and E, which bind different DNA sequences, interact with different partners and thus have a different set of target genes. Therefore, it is inaccurate to suggest that SOX2 expression increase is compensatory. The 2 proteins are not performing the same function, thus SOX2 can never compensate for SOX9 absence in the genetic sense. In agreement, the author report a cellular phenotype in absence of SOX9 despite an increase in SOX2 expression, therefore there is no compensation, and this term should be replaced.
We appreciate your comments on our findings. Nonetheless, we reported the compensatory SOX2 over SOX9 expression based on other research articles (listed Domenici et al. A Sox2-Sox9 signaling axis maintains human breast luminal progenitor and breast cancer stem cells. 2019), where they confirm a compensatory stem-loop behavior among SOX2 and SOX9 by DNA binding chromatin immunoprecipitation. To a better understanding, we modified the abstract (See page 2, line 40) and mini abstract sections (See page 3, line 51). Also, we modified the discussion section to avoid confusions about SOX2 and SOX9 target genes (See page 13, line 290).

The discussion is lengthy, with correlations and differences with different studies on aspects of the phenotype listed where the authors finally suggest that differences in culture conditions prevent meaningful comparisons. Maybe a shorter discussion focusing on meaningful aspects of the study in relation with what is known in the field would be better. As the authors mention in the introduction, the role of SOX9 in CRC has been previously studied, and maybe it would be best to restrict the discussion there.
Attending your suggestions, we already modified discussion section deleting extra information that possibly confused to the reader.
Line 212, “aquiline”, please revise.
The term “aquiline” was eliminated to avoid confusions.

Reviewer B

Notes:
• Role of Sox9 in CSCs in many cancers already well established. Sox9 previously described as a tumor suppressor in CRC. Novelty is questionable. Novelty in our research relies on that we performed a complete CSCs markers characterization of the most employed CRC cell lines, also, we performed the first SOX9 abolition to evaluate to the CSCs population in CRC cell lines since SOX9 is an important transcription factor in the development of CRC. Besides, morphological changes have never been reported by SOX9 absence in CRC. Further, the compensatory mechanism between SOX9 and SOX2 have been never reported in colorectal cancer only in breast cancer cells (listed Domenici et al. A Sox2-Sox9 signaling axis maintains human breast luminal progenitor and breast cancer stem cells. 2019). These are some of our findings to mention.

• Does not explain the criteria required that meets the stem phenotype and why it matters.
We already added in the introduction section the importance of the stemness phenotype in CRC cells, also we mentioned the characteristics of both, the surface markers and the TFs related to CSCs (See page 4, line 60 to 69)
• Table 1 could be a figure like Figure 1.
We appreciate your suggestions but we prefer to maintain the original format due to we needed to add another figure with more relevant information.
• In Figure 4 they don't explain the significance of having an increased # of spheres in SOX9-KO but a decrease in size vs having the HCT116 line produced less # of spheres but bigger in size. How was cell density quantified – text states a significant lower cell density.
We already rearrange the text with the importance of this effect in the results section (See page 10, line 222-226 and page 17, line 386-391). We also added in material and methods section the procedure for cell density determination as well as spheres counting (See page 8, line 160-162).
• The fact that cell morphology is changed (phalloidin) – radically – even before sphere formation, makes any changes in sphere formation seem irrelevant. Cells with disrupted cytoskeleton make different structured spheres.
We appreciate your suggestions; we wrote the possible cause by which SOX9 KO spheroid presented more labile-ness (See page 11, line 237-239).
• EMT is tested by marker gene with no mention of other assays that actual test mobility, migration etc. We only restricted the information related to EMT genes expression changes, we attempt to describe these changes (conferred by functional analysis as migration) in another research article (Below is an example of the migration test carried out) between HCT116 *vs* KO cells).



• Analysis on differentiation is quite superficial.
We already add information in order to make this analysis clearer (See page 8, line 160).
• Overall language, format and sentence structure of the paper does not flow properly. For instance, would be better to state what qualities in a cell line would be required for the planned experiments, then show results then conclude that HCT116 cells are the best – rather than use a word like "settled".
As presented along the text, we carefully write sentences by make coherence sequence, we

talked about the importance of this transcription factor in CRC characteristics, their importance in the maintenance of CSCs properties as well as its relevance in clinical characteristics. We mentioned the most important characteristics (phenotypical) in the mentioned CRC cell lines, their comparison leads us to stablish the methodology by selecting HCT116 over the other cell lines since it showed the more stemness properties (related to surface markers and transcription factor levels). However, we changed the text where we defined the HCT116 as the best cell line. (See page 9, line 183 and page 13, line 276-278).

• Sometimes detail is lacking for instance analysis mentioned – but not told what kind (IFC presumably).

We already reviewed all the material and methods section to verify that all the procedures were well described.

• Typo – a figure 5F is mentioned.

We modified all the sequence of the figures in the text including errors.

Just seems very superficial and not very interesting. Not sure who would read this report.