

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Using a theranostical targeted nanomedicine platform, this study demonstrates the key oncogenic role of PBOV1, a human protein coding gene with a 2501 bp single exon-mRNA, in the Hepatocellular Carcinoma (HCC). The proposed theranostical nanomedicine is composed of a single chain antibody for epidermal growth factor receptor (scAb-EGFR) which is conjugated to a copolymer of polyethyleneglycol-polyethylenimine (PEG-PEI) complexing small nanoparticles of iron oxide (SPION). These nanoparticles (scAb-EGFR-PEG-PEI-SPION) were loaded with either PBOV1 siRNA for gene silencing or with PBOV1-pDNA for gene overexpression. The HCC cell transfection of scAb-EGFR-PEG-PEI-SPION nanoparticles was demonstrated, resulting in the down regulation or the overexpression of PBOV1 gene, depending if PBOV1-siRNA or PBOV1-pDNA were polyplexed with the scAb-EGFR targeted nanocarrier. Then, the relationship between PBOV1 expression levels and hepatocarcinoma cell growth and metastasis was demonstrated both in vitro and in vivo using an orthotopic liver tumor model and a model of lung HCC metastasis. The in vivo results were supported by in vivo biodistribution studies using MRI and near-infrared-fluorescence imaging. Finally, it was nicely demonstrated that HCC stemness may be regulated by scAb-EGFR-PEG-PEI-SPION nanoparticles both in vitro and in vivo.

The construction of targeted theranostic nanoparticles based on functionalized PEG-PEI for complexation of siRNA or pDNA is not a novel concept. Also, previous studies have already demonstrated that PBOV1 overexpression promoted cancer cell proliferation in prostate, breast but also recently in ovarian cancer (Wang et al., *Oncology Reports*, 2016). But the novelty of the present study resides in the demonstration of the oncogenic role of PBOV1 in growth and metastasis of Hepatocellular carcinoma by using the EGFR targeted nanoparticles. Indeed, the oncogenic role of PBOV1 in HCC has not been reported before and the authors have nicely highlighted the molecular mechanisms affecting Wnt/beta-catenin pathway. The reviewer acknowledges the huge amount of interesting and relevant experimental data collected both in vitro and in vivo. The results obtained are comprehensive, very convincing and they support the conclusions of this publication. Thus, concerning the improvement of the paper from a technical point of view, the reviewer has only few comments below, mainly related to toxicological data. Specific comments

- The introduction is a little bit too long and could be shortened to better focus on the aim of the study
- Although the authors have performed some liver and kidney acute toxicity experiments (figure 5), it is widely accepted that due to its cationic character, there are toxicity issues related to the intravenous administration of PEI and PEI derivatives. Also, the PEG-PEI copolymer is not a biodegradable material which raises queries about the elimination of this polymer and possible accumulation. Excretion investigations should be performed and toxicity studies performed with higher doses and repeated administration of scAb-EGFR-PEG-PEI-SPION nanoparticles which are needed for efficient therapy. Otherwise, a fair discussion should be added to clearly restrict the use of such nanocarrier in the clinic.
- Figure 5: Dose and schedule of administration are lacking in the figure legend. But from supplementary information, it seems that toxicity experiments were performed by injecting scAb-EGFR-PEG-PEI-SPION only once, when for the pharmacological efficacy study in figure 6, 20 doses were injected iv, once a day, during 20 days. Toxicity assays should have been performed using at least the same injection schedule with eventually higher doses (as performed usually to check the toxicity issues of a drug in pre-clinical assays).
- page 9, line 200 "CTX group" should be replaced by "CTS group".
- Concerning the in vivo pharmacological efficacy experiments, in Figure 6 (b) and (d), only the endpoint of the liver tumor weight or number of lung tumor metastasis are given, but a kinetic of the tumor growth and of the number of tumor metastasis should be given.
- In Figure 6, the injected doses and schedule of administration are lacking in the figure caption. From the supplementary information, it seems that the nanoparticles bearing the siRNA were injected once a day, during 20 days which is a lot of administrations and emphasizes the

toxicological issues mentioned above.

Reviewer #2 (Remarks to the Author):

This manuscript entitled "How PBOV1 promotes hepatocellular carcinoma: the oncogenic role identified by nanomedicines for effective gene therapy" reports a EGFR specific single chain antibody targeted nonviral nanocarrier for co-delivery of PBOV1 silencing or promoting plasmid and superparamagnetic iron oxide nanocrystals. The results of this study verify that this theranostic nucleic acid nanomedicine possesses great potential for PBOV1 targeted gene therapy and MRI diagnosis of hepatocellular carcinoma. However, the novelty of this study is limited since similar biomaterial formulation has been developed in some published papers (e.g., Guo, Y., et al. 2016, *Hepatology* 63, 1240-1255). Also, there are inadequate explanations of the rationale for the study design. And the following are some suggestions to improve this manuscript.

Page 9 (Figure 3e) "After 1h cell incubation, the uptake amount of non-targeted..." There should be more time points here to compare the intracellular uptake of EGFR-target vector with non-target vector.

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Reviewer #3 (Remarks to the Author):

The authors of the manuscript „How PBOV1 promotes hepatocellular carcinoma: the oncogenic role identified by nanomedicines for effective gene therapy“ evaluate the functions of PBOV1 (prostate and breast cancer overexpressed 1). Briefly, they demonstrate that PBOV1 is overexpressed in human HCC as compared to adjacent non-tumorous tissues and that PBOV1 expression correlates with poor overall and disease free survival. In the next step, they use a modified approach to deliver PBOV1-encoding plasmid DNA or respective siRNAs in a murine xenotransplantation HCC model by the means of nanoparticles which are directed specifically to EGFR-expressing cells. Overexpression of PBOV1 resulted in increased tumor growth characterized by reduced apoptosis, increased angiogenesis as well as metastasis and overexpression of Survivin and Cyclin D1, while caspase 3 cleavage was reduced. This phenotype could be reversed by using PBOV1-directed siRNAs. Moreover, the authors claim that PBOV1 expression correlates with EMT as well as "tumour stem cell"-like features such as sphere formation and expression of OCT4, NANOG and beta-Catenin.

The manuscript is relatively well written (although some minor editorial and stylistic modifications and corrections are warranted) and most of the individual experiments are well performed. However, the individual experiments are not connected so well to "build a story" and the manuscript is therefore difficult to understand, in particular since (too) many different aspects of targeted tumor therapy (including its detection by MRI) and PBOV1-mediated effects on hepatoma cell biology are addressed. Moreover, some of the presented findings remain descriptive and could be studied in more depth and detail as described below.

1. The authors claim that they use a novel "nanomedicine" to target oncogenes in HCC. This

approach has previously been described (PMID 26680504) and the present manuscript reiterates many of the Figures presented by Guo et al. in Hepatology– simply with another oncogene. If I understand correctly, the major modification as compared to the previous report is a more targeted delivery by including EGFR-directed antibodies. Therefore, the authors should elaborate more on the impact of EGFR-targeting in comparison to the previously used particles on tumor cell delivery in vitro and in vivo. Moreover, the MRI visualization is a nice feature, but does not add significant insights to the authors' conclusions. How can tumor therapy be optimized by using MRI in this setting? In keeping with this notion, the scheme provided in Figure 1 reiterates assumptions from the text and should be omitted.

2. Although the authors clearly show that PBOV1 expression is increased in human HCC and correlates with tumor prognosis, the exact mechanism of action of PBOV1 remains unclear. The immunohistochemical staining pattern of PBOV1 appears to be quite diverse with cytoplasmic staining in some tumors and cytoplasmic as well as nuclear staining in others. The authors should provide pictures with more magnification and should address the question whether PBOV1 expression is indeed restricted to tumor cells or whether infiltrating immune cells etc. also stain positive.

It is shown that PBOV1 promotes cell proliferation in vitro. Therefore, tumor cell proliferation (e.g. by BrDU staining) should also addressed in xenotransplant tumors (Fig. 6c). The authors state in the Discussion that they “revealed the molecular mechanisms of PBOV1”. However, the exact functions of PBOV1, its target genes etc. are not sufficiently clear (at least to me). Can any conclusions on its mechanistic functions be drawn from its protein structure? What regulates its expression in tumor cells? The presented differences on CCND1, Survivin and caspase 3 as well as the resulting differences on apoptosis, proliferation etc. may simply be secondary events. Therefore, a more thorough description of differences in gene expression would be useful to better understand the real molecular functions of PBOV1.

3. The conclusion that PBOV1 affects EMT and cancer stem cell biology is speculative, since EMT has not been addressed by using the adequate epithelial and mesenchymal markers and immortalized hepatoma cells may not be a good tool to study EMT. In keeping with this notion, the findings on CSC biology simply rely on descriptive markers of OCT4 and NANOG expression while there is no evidence that true stem tumor cell biology is really affected by PBOV1.

4. The discussion simply reiterates the results section and a more critical discussion of the findings in comparison to the literature needs to be included.

Response to Reviewer #1 comments

Reviewer #1 (Remarks to the Author):

Using a theranostical targeted nanomedicine platform, this study demonstrates the key oncogenic role of PBOV1, a human protein coding gene with a 2501 bp single exon-mRNA, in the Hepatocellular Carcinoma (HCC). The proposed theranostical nanomedicine is composed of a single chain antibody for epidermal growth factor receptor (scAb-EGFR) which is conjugated to a copolymer of polyethyleneglycol-polyethylenimine (PEG-PEI) complexing small nanoparticles of iron oxide (SPION). These nanoparticles (scAb-EGFR-PEG-PEI-SPION) were loaded with either PBOV siRNA for gene silencing or with PBOV-pDNA for gene overexpression. The HCC cell transfection of scAb-EGFR-PEG-PEI-SPION nanoparticles was demonstrated, resulting in the down regulation or the overexpression of PBOV gene, depending if PBOV-siRNA or PBOV-pDNA were polyplexed with the scAb-EGFR targeted nanocarrier. Then, the relationship between PBOV expression levels and hepatocarcinoma cell growth and metastasis was demonstrated both in vitro and in vivo using an orthotopic liver tumor model and a model of lung HCC metastasis. The in vivo results were supported by in vivo biodistribution studies using MRI and near-infrared-fluorescence imaging. Finally, it was nicely demonstrated that HCC stemness may be regulated by scAb-EGFR-PEG-PEI-SPION nanoparticles both in vitro and in vivo.

The construction of targeted theranostic nanoparticles based on functionalized PEG-PEI for complexation of siRNA or pDNA is not a novel concept. Also, previous studies have already demonstrated that PBOV1 overexpression promoted cancer cell proliferation in prostate, breast but also recently in ovarian cancer (Wang et al., *Oncology Reports*, 2016). But the novelty of the present study resides in the demonstration of the oncogenic role of PBOV1 in growth and metastasis of Hepatocellular carcinoma by using the EGFR targeted nanoparticles. Indeed, the oncogenic role of PBOV1 in HCC has not been reported before and the authors have nicely highlighted the molecular mechanisms affecting Wnt/beta-catenin pathway. The reviewer acknowledges the huge amount of interesting and relevant experimental data collected both in vitro and in vivo. The results obtained are comprehensive, very convincing and they support the conclusions of this publication. Thus, concerning the improvement of the paper from a technical point of view, the reviewer has only few comments below, mainly related to toxicological data.

Specific comments

- The introduction is a little bit too long and could be shortened to better focus on the aim of the study

Response: The reviewer's suggestion is highly appreciated. As suggested, we have shortened the introduction to better focus on the aim of the study.

- Although the authors have performed some liver and kidney acute toxicity experiments (figure 5), it is widely accepted that due to its cationic character, there are toxicity issues related to the intravenous administration of PEI and PEI derivatives. Also, the PEG-PEI copolymer is not a biodegradable material which raises queries about the elimination of this polymer and possible accumulation. Excretion investigations should be performed and toxicity studies performed with higher doses and repeated administration of scAb-EGFR-PEG-PEI-SPION nanoparticles which are needed for efficient therapy. Otherwise, a fair discussion should be added to clearly restrict the use of such nanocarrier in the clinic.

Response: We thank the reviewer for the valuable toxicity concerns about PEG-PEI copolymer due to the cationic and nonbiodegradable characters. As suggested, we investigated the excretion and studied the toxicities with higher doses and repeated administration of scAb-EGFR-PEG-*g*-PEI-SPION nanoparticles which are needed for efficient therapy. Renal excretion was not detectable, but the fluorescent carrier was found in gallbladder, implying a hepatic metabolism pathway (Supplementary Fig. 9b). Liver toxicities increased along with increase in dose, and renal toxicities were not detected even at 5 × normal dose (once a day for 20 d *via* tail vein) (Supplementary Fig. 9c,d). As suggested, we added a fair discussion to clearly restrict the use of such nanocarrier in the clinic. We want to note, as pointed out by the reviewer, the main purpose of the present study is to explore the oncogenic role of PBOV1 in growth and metastasis of Hepatocellular carcinoma by using the EGFR targeted nanoparticles. The finding of this study lays a foundation for further developing more applicable siRNA nanomedicines based on biodegradable carriers

- Figure 5: Dose and schedule of administration are lacking in the figure legend. But from supplementary information, it seems that toxicity experiments were performed by injecting scAb-EGFR-PEG-PEI-SPION only once, when for the pharmacological efficacy study in figure 6, 20 doses were injected iv, once a day, during 20 days. Toxicity assays should have been performed using at least the same injection schedule with eventually higher doses (as performed usually to check the toxicity issues of a drug in pre-clinical assays).

Response: Thanks for reviewer's comments. The injected doses and schedule of administration were described in the experimental methods and Fig. 4h. In the previous version, scAb-EGFR-PEG-PEI-SPION was injected only once for investigating serum function markers, but was injected once a day for 20 d for liver and kidney H&E staining experiment (previously Fig. 5d,e, now Fig. 4g). As suggested, we performed additional toxicity assay using the same injection schedule with $5 \times$ dose (Supplementary Fig. 9c,d). Clear liver toxicity was detected at this high dose (still no renal toxicity detected). In comparison, at $1 \times$ dose for achieving ideal therapeutic effect, only limited liver toxicity was shown (Fig. 4f, Supplementary Fig. 9c,d).

- page 9, line 200 “CTX group” should be replaced by “CTS group”.

Response: Thank the review for pointing out this typo. As suggested, “CTX group” was replaced by “CTS group”.

- Concerning the in vivo pharmacological efficacy experiments, in Figure 6 (b) and (d), only the endpoint of the liver tumor weight or number of lung tumor metastasis are given, but a kinetic of the tumor growth and of the number of tumor metastasis should be given.

Response: Thanks for reviewer's suggestions. As suggested, kinetic of the liver orthotopic or subcutaneous tumor growth (Fig. 5b,g,6f, Supplementary Fig.7b) and the number of tumor metastasis (Fig. 6b) were given in the revised manuscript.

- In Figure 6, the injected doses and schedule of administration are lacking in the figure caption. From the supplementary information, it seems that the nanoparticles bearing the siRNA were injected once a day, during 20 days which is a lot of administrations and emphasizes the toxicological issues mentioned above.

Response: Thank the reviewer for the valuable concern about the toxicological issues again. We performed additional toxicity studies and the data are included in the revised manuscript (Fig. 4f,g, Supplementary Fig. 9c,d). The injected doses and schedule of administration were described in the experimental methods (Fig. 4h), and we added the information to figure legend in the revised manuscript.

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This manuscript entitled “How PBOV1 promotes hepatocellular carcinoma: the oncogenic role identified by nanomedicines for effective gene therapy” reports a EGFR specific single chain antibody targeted nonviral nanocarrier for co-delivery of PBOV1 silencing or promoting plasmid and superparamagnetic iron oxide nanocrystals. The results of this study verify that this theranostic nucleic acid nanomedicine possesses great potential for PBOV1 targeted gene therapy and MRI diagnosis of hepatocellular carcinoma. However, the novelty of this study is limited since similar biomaterial formulation has been developed in some published papers (e.g., Guo, Y., et al. 2016, Hepatology 63, 1240-1255). Also, there are inadequate explanations of the rationale for the study design. And the following are some suggestions to improve this manuscript.

Page 9 (Figure 3e) “After 1h cell incubation, the uptake amount of non-targeted...” There should be more time points here to compare the intracellular uptake of EGFR-target vector with non-target vector.

Response: Thanks for the helpful comments. As suggested, we added more time points to compare the intracellular uptake of EGFR-target vector with non-target vector (Fig.2e).

Page 16 (Figure 5b) “Moreover, the NIRF signal was strong in tumor area of the...” Please check the NIRF imaging of mice after 0, 0.5, 2, 6, 12, 24h of nanomaterials injection.

Response: The reviewer's comments are highly appreciated. As suggested, we checked the NIRF imaging of mice after 0, 0.5, 2, 6, 12, 24 h of nanomaterials injection (Supplementary Fig.10, Fig. 4b). Consistent with the MRI results (Fig. 4a), tumor accumulation of nanomedicine was detected from 0.5 h after tail vein injection.

Page 16 (Figure 5c) “Prussian blue staining for iron deposition in tumor tissue was highly supportive...” Please check the Fe depositions in normal liver tissues.

Response: Thanks for reviewer's suggestions. As suggested, we checked the Fe depositions in normal liver tissues (Supplementary Fig. 9a). Consistent with imaging

study results (Fig. 4a,b, Supplementary Fig. 10), Fe depositions were detected and increased at prolonged time in normal liver tissues (6 h vs 2 h).

Page 15 (Figure 5) To evaluate the sensitivity and specificity of single chain EGFR specific Ab, please provide the *in vivo* biodistribution data (including all the organs and tissues).

Response: Thanks for reviewer's comments. As suggested, the *in vivo* biodistribution data (including major organs and tissues) were provided (Fig.4d,e).

Page 18 (Figure 6) Please add a subcutaneous tumor model here to observe the tumor inhibition during the treatment.

Response: The reviewer's suggestion is highly appreciated. The tumor inhibition during the treatment was observed in a subcutaneous tumor model (Fig. 5g,6f,g, Supplementary Fig. 7a,b), which was performed to show the effect of nanomedicine treatment on tumorigenesis ability.

Reviewer #3 (Remarks to the Author):

The authors of the manuscript "How PBOV1 promotes hepatocellular carcinoma: the oncogenic role identified by nanomedicines for effective gene therapy" evaluate the functions of PBOV1 (prostate and breast cancer overexpressed 1). Briefly, they demonstrate that PBOV1 is overexpressed in human HCC as compared to adjacent non-tumorous tissues and that PBOV1 expression correlates with poor overall and disease free survival. In the next step, they use a modified approach to deliver PBOV1-encoding plasmid DNA or respective siRNAs in a murine xenotransplantation HCC model by the means of nanoparticles which are directed specifically to EGFR-expressing cells. Overexpression of PBOV1 resulted in increased tumor growth characterized by reduced apoptosis, increased angiogenesis as well as metastasis and overexpression of Survivin and Cyclin D1, while capsase 3 cleavage was reduced. This phenotype could be reversed by using PBVO1-directed siRNAs. Moreover, the authors claim that PBOV1 expression correlates with EMT as well as "tumour stem cell"-like features such as sphere formation and expression of OCT4, NANOG and beta-Catenin.

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1. The authors claim that they use a novel "nanomedicine" to target oncogenes in HCC. This approach has previously been described (PMID 26680504) and the present manuscript reiterates many of the Figures presented by Guo et al. in Hepatology– simply with another oncogene. If I understand correctly, the major modification as compared to the previous report is a more targeted delivery by including EGFR-directed antibodies. Therefore, the authors should elaborate more on the impact of EGFR-targeting in comparison to the previously used particles on tumor cell delivery in vitro and in vivo. Moreover, the MRI visualization is a nice feature, but does not add significant insights to the authors' conclusions. How can tumor therapy be optimized by using MRI in this setting? In keeping with this notion, the scheme provided in Figure 1 reiterates assumptions from the text and should be omitted.

Response: The reviewer's comments are highly appreciated. As suggested, we conducted more experiments to compare the impact of EGFR-targeting with that of folate-targeting on tumor cell delivery. No much difference was observed *in vitro*. However, EGFR-targeted *p*DNA nanomedicine showed better effect than the folate-targeted one in promoting gene delivery to HCC cells *in vivo* (Supplementary Fig. 8a), resulting in more effective up-regulation of PBOV1 expression (Supplementary Fig. 8b,c).

Non-invasive imaging was important for monitoring the nanomedicine fate *in vivo*. Compared to the NIRF imaging only showing a rough location of fluorescent agents (Fig. 4b, Supplementary Fig. 10), MRI with high spatial resolution may provide more accurate information on the organ/tumor distribution of MRI-visible nanomedicines (Fig. 4a,5d). Moreover, it is well-known that MRI has the potential to non-invasively detect the tumor size and number (Fig. 5c,d). In this context, MRI may be used to assist a rational design of dose and schedule of administration. Indeed, the therapeutic effect of a nanomedicine was not related to its MRI-visible function. As suggested, the scheme provided in previously Figure 1 was omitted.

2. Although the authors clearly show that PBOV1 expression is increased in human HCC and correlates with tumor prognosis, the exact mechanism of action of PBOV1 remains unclear. The immunohistochemical staining pattern of PBOV1 appears to be quite diverse with cytoplasmic staining in some tumors and cytoplasmic as well as nuclear staining in others. The authors should provide pictures with more magnification and should address the question whether PBOV1 expression is indeed restricted to tumor cells or whether infiltrating immune cells etc. also stain positive.

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The authors state in the Discussion that they “revealed the molecular mechanisms of PBOV1”. However, the exact functions of PBOV1, its target genes etc. are not sufficiently clear (at least to me). Can any conclusions on its mechanistic functions be drawn from its protein structure? What regulates its expression in tumor cells? The presented differences on

CCND1, Survivin and caspase 3 as well as the resulting differences on apoptosis, proliferation etc. may simply be secondary events. Therefore, a more thorough description of differences in gene expression would be useful to better understand the real molecular functions of PBOV1.

Response: Thanks for reviewer's helpful comments. As suggested, we provided pictures with more magnification (Fig. 5f 2nd panel, Supplementary Fig. 11). HCC cells were stained positive whereas stromal cells (including immune cells and fibrocytes) were not stained positive, which confirmed that PBOV1 expression was restricted to tumor cells (Supplementary Fig. 11).

As suggested, BrdU staining and Ki67 staining were performed to further show tumor cell proliferation in xenotransplant tumors (Fig. 5f).

Per reviewer's suggestion, we retrieved protein structure of PBOV1 in Conserved Domain Database (CDD) of NCBI, however there is no conserved domain was identified. According to a previous report, PBOV1 lacks orthologues in non-primate genomes and PBOV1 protein-coding sequence is human-specific and has originated *de novo* in the primate evolution through a series of frame-shift and stop codon mutations⁴. The authors also did bioinformatics analysis of PBOV1 protein and found no functional features of PBOV1. Thus, it may be not so easy to draw any conclusion on its mechanistic functions from its protein structure.

Then, to reveal the underlying mechanisms of PBOV1 on promoting HCC stemness and EMT, mass spectrometry was performed. Surprisingly, β -catenin, which has been well proved to play pivotal roles in regulation of EMT and stemness in kinds of tumors, was identified as a binding partner of PBOV1 (Fig. 7a, b). Co-immunoprecipitation and immunostaining further confirmed the interaction (Fig. 7c,d). Moreover, it was found that PBOV1 activates β -catenin signaling (Fig. 7e) by decreasing phosphor- β -catenin (Ser33/37/Thr41) expression and promoting nuclear translocation of β -catenin *in vitro* and *in vivo* (Fig. 7f,g,i), which was achieved by impairing GSK-3 β binding (Fig. 7h) and subsequently phosphorylating β -catenin (Fig. 7j).

PBOV1 was firstly found to be overexpressed in prostate, breast, and bladder cancer⁵. The following investigation showed that PBOV1 was elevated in tumors of

16 different tissues and organs, including liver⁴. It has been previously shown that PBOV1 expression in breast cancer and prostate cancer cells is positively regulated by estrogen⁶ and dihydrotestosterone⁵, respectively, which indicated that the expression of PBOV1 might be hormone-dependent in these tumors. Moreover, according to the research of Nikolay Samusik et al., the promoter of PBOV1 is CpG poor, thus CpG methylation could not be important in PBOV1 regulation. However, they reported that FOXA1, FOXA2, C/EBP transcription factor family and Hedgehog pathway might involve in regulating expression of PBOV1⁴. In the last decades, microRNA has been widely proved to modulate gene expression. It is highly likely that dysregulated microRNAs also participate in regulating PBOV1 expression. This study focuses on the effects of PBOV1 on HCC progression and the potential clinical application of PBOV1 as a therapeutic target, and we hope that our reversion adds a more thorough description of differences in gene expression for better understandings of the PBOV1 functions in HCC.

3. The conclusion that PBOV1 affects EMT and cancer stem cell biology is speculative, since EMT has not been addressed by using the adequate epithelial and mesenchymal markers and immortalized hepatoma cells may not be a good tool to study EMT. In keeping with this notion, the findings on CSC biology simply rely on descriptive markers of OCT4 and NANOG expression while there is no evidence that true stem tumor cell biology is really affected by PBOV1.

Response: The reviewer's comments are highly appreciated. The idea that epithelial cells can downregulate epithelial characteristics and acquire mesenchymal characteristics arose in the early 1980s from observations made by Elizabeth Hay⁷, who described epithelial to mesenchymal phenotype changes in the primitive streak of chick embryos⁸. With the development of research on EMT, it was parsed into three subtypes EMT based on the context under which they occur. Type 1 involves primitive epithelial cells transitioning to motile mesenchymal cells as part of gastrulation and primitive neuroepithelial cells generating migrating neural crest cells. Type 2 involves secondary epithelial or endothelial cells transitioning to resident tissue fibroblasts. Type 3 involves epithelial carcinoma cells in primary nodules transitioning to metastatic tumor cells^{9,10}. In recent years, EMT has been

widely investigated in cancer biology *in vitro* and *in vivo* and has been proved to be an early step of metastasis and associate with stem cell properties acquisition of cancer cells^{11,12}. Cancer stem cells (CSCs) are tacitly believed to have acquired the molecular armaments of normal stem cells: CSCs can renew themselves, and they are built to last a lifetime, to be resilient to electromagnetic and chemical insults, to be able to slumber for prolonged periods of time and to colonize other parts of the body. It was reported that CSCs express pluripotent markers, such as OCT4, NANOG, KLF4 and so on¹³. Except that, EpCAM-positive cells, CD133-positive cells or the side population cells are also characterized as HCC CSCs¹⁴⁻¹⁶.

In the current study, we find that PBOV1 induced EMT of HCC cells as indicated by losing epithelial markers E-cadherin and α -Catenin, and acquiring mesenchymal markers Vimentin and N-cadherin (Fig. 3f,g, Fig. 6c) and accompanied with increased migration and invasion activities (Fig. 3e, Fig. 6a,b). Moreover, overexpression of PBOV1 enhanced, while knock down of PBOV1 weakened, stem cell properties of HCC cells as indicated by the expression of pluripotent markers OCT4, NANOG and c-myc (Fig. 6e,h), percentage of side population cells, percentage of CD133 and EpCAM positive cells, tumor sphere formation ability and subcutaneous tumor incidence (fig. 6d,e,f,g, Supplementary Fig. 7a,b).

All in all, these results indicated that PBOV1 enhanced EMT and stemness of HCC.

4. The discussion simply reiterates the results section and a more critical discussion of the findings in comparison to the literature needs to be included.

Response: Thanks for the helpful comments. As suggested, we modified the discussion section.

Once again, we thank the referees for their helpful suggestions and critiques. We do hope that the substantially revised manuscript would meet your requirements for a further consideration.

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have significantly improved the overall quality of the paper and additional experiments, well conducted, have answered all my toxicity concerns. I therefore recommend the publication of this interesting study.

Reviewer #2 (Remarks to the Author):

While the construction of PEG-PEI conjugated SPION for targeted gene therapy is not a novel concept, lots of in-vitro and in-vivo data demonstrate that the nanoparticles loading siRNA specifically target PBOV1 have emerged as a powerful strategy in hepatocellular cancer treatment. I think the authors have answered all the questions clearly overall. Here I also suggest some minor revisions to this manuscript.

1. How about the lifetime of the nanoparticles loading pDNA/siRNA in blood?
2. (Fig. 3a, Fig. 5c and 5g, and Fig. 6f) Please provide more detailed statistical data of comparisons of tumor volumes.

Reviewer #3 (Remarks to the Author):

Most of my concerns have been sufficiently addressed in the revised version of the manuscript.

Point-by-point response to the referee comments

Response to Reviewer #1 comments

Reviewer #1 (Remarks to the Author):

The authors have significantly improved the overall quality of the paper and additional experiments, well conducted, have answered all my toxicity concerns. I therefore recommend the publication of this interesting study.

Response: We thank you for your positive comments. We are glad that the revision answered your toxicity concerns. We really appreciate for your help in improving the quality of the manuscript.

Response to Reviewer #2 comments

Reviewer #2 (Remarks to the Author):

While the construction of PEG-PEI conjugated SPION for targeted gene therapy is not a novel concept, lots of in-vitro and in-vivo data demonstrate that the nanoparticles loading siRNA specifically target PBOV1 have emerged as a powerful strategy in hepatocellular cancer treatment. I think the authors have answered all the questions clearly overall. Here I also suggest some minor revisions to this manuscript.

1. How about the lifetime of the nanoparticles loading pDNA/siRNA in blood?

Response: We thank the reviewer for the valuable concern about lifetime of nanomedicine in blood. We conducted additional experiments to determine the concentrations of nanomedicines in the blood using the fluorescence intensity assay [see: Li J, Yu X, Wang Y, Yuan Y, Xiao H, Cheng D, Shuai X. Adv Mater. 2014 Dec 23;26(48):8217-24. doi: 10.1002/adma.201403877]. We found that the $t_{1/2}$ of nanomedicines used in this study was about 4 h (Supplementary Fig. 12).

2. (Fig. 3a, Fig. 5c and 5g, and Fig. 6f) Please provide more detailed statistical data of comparisons of tumor volumes.

Response: We thank the reviewer for the valuable concern about statistical analysis. We analyzed data in Fig. 3a, Fig. 5c and 5g, and Fig. 6f by Two-way ANOVA and Student's *t*-test to provide detailed statistical results. The results were shown in the figures.

Response to Reviewer #3 comments

Reviewer #3 (Remarks to the Author):

Most of my concerns have been sufficiently addressed in the revised version of the manuscript.

Response: Thank you very much for your positive comments on our revisions. We are glad that the revision sufficiently addressed your concerns. We really appreciate for your help in improving the quality of the manuscript.