Sestrin2 reduces the cancer stemness via the Wnt/β-catenin signaling in colorectal cancer

Jinlai Wei
The First Affiliated Hospital of Chongqing Medical University
xiangru zheng (xruzheng@outlook.com)
The Third Affiliated Hospital of Chongqing Medical University (Gener Hospital) https://orcid.org/0000-0003-1170-943X
Wenjun Li
The third affiliated Hospital of Chongqing Medical University (Gener Hospital)
Xiaoli Li
Chongqing Medical University
Zhongxue Fu
The First Affiliated Hospital of Chongqing Medical University

Research Article

Keywords: Sestrin2, Colorectal cancer, Wnt/β-catenin, cancer stemness, therapeutic target.

Posted Date: August 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-695161/v1

Version of Record: A version of this preprint was published at Cancer Cell International on February 11th, 2022. See the published version at https://doi.org/10.1186/s12935-022-02498-x.

Abstract

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women in China. In previous studies, Sestrin2 was demonstrated to have functions in CRC. However, the relationship between Sestrin2 and cancer stemness has not been reported. To investigate the contribution of Sestrin2 in CRC, we performed bioinformatics analysis of The Cancer Genome Atlas datasets and found that Sestrin2 was downregulated in CRC. Using a lentivirus vector, we verified that Sestrin2 suppressed CRC cell proliferation, migration, and colony formation. Furthermore, sphere formation, flow cytometry, quantitative PCR, and Western blot analysis verified the effect of Sestrin2 on the cancer stem ness, including the expression of cluster of differentiation 44, octamer-binding transcription factor 4, sex-determining region Y-box 2, CXC chemokine receptor 4, and the Wnt pathway downstream factors β-catenin and c-Myc. Consistently, the Wnt pathway activator BML-284 partially rescued the effects of Sestrin2 on the expression of proteins related to the cancer stemness. Furthermore, in a mouse xenoplant model, tumors with Sestrin2 were significantly reduced in size with corresponding changes in the cancer stemness. Collectively, our results suggest that Sestrin2 inhibits CRC cell progression by downregulating the Wnt signaling pathway. Thus, Sestrin2 may be a promising therapeutic target for CRC.

Background

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women in China [1]. Standard anti-cancer therapies for CRC include surgery and chemotherapy, but the survival rate is still poor. The failure of standard anti-cancer treatments is due to cancer stem cell (CSC) [2].CSCs are tumour cells that have properties of self-renewal, tumour initiation capacity, and long-term repopulation potential [3]. Targeting CSCs would be a promising therapeutic method. Therefore, investigating on CSCs to find new molecules and mechanisms in CRC would supply novel therapeutic targets.

At present, A variety of molecules are found to participate in CSC. Many cell surface proteins are used as CRC markers for different derived tumours, for example, cluster of differentiation 34-positive (CD34+)/CD38- for leukaemia cells, CD13/CD45/CD90 for liver cancer, CD117/CD90/epithelial cell adhesion molecule for lung cancer [4, 5], and CD44 for colon and gastric cancers [6]. The stem cell state is governed by stem cell factors such as the four Yamanaka factors: sex-determining region Y-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), Kruppel-like factor 4, and c-Myc [7].

Wnt signalling is one of the key pathways that regulate development and stemness in cancer [8]. The function of the Wnt pathway in CSCs is commonly accepted and depends on the amount of β-catenin in the cytoplasm [9, 10]. β-catenin is able to activate not only proliferation and transmission factors, such as c-Myc, c-Jun, CCND1 (the gene encoding cyclin D1), but also epidermal growth factor receptor, like CD44 and CD133.

Sestrin family members including Sestrin1, Sestrin2, and Sestrin3 can be induced by cellular stresses, such as ER stress, DNA damage and ROS accumulation[11–13]. Among sestrin family, Sestrin2 has

shown regulating activities in some cancers [14]. Knocking down Sestrin2 in non-small cell lung cancer cells resulted in reduced lung cancer progression [15]. Down-regulation of Sestrin2 promoted cell proliferation, migration, and ROS production in endometrial cancer cells [16]. In CRC, our previous study demonstrated that there is a lower expression of Sestrin2 than in normal colorectal epithelial [17]. Other studies have shown that Sestrin2 has many other functions in CRC; for example, Sestrin2 is involved in inducing apoptotic process of HCT116 CRC cells [18], and its overexpression inhibits the migration, invasion, and growth of CRC [19]. However, the relationship between Sestrin2 and CRC stemness has not been previously reported.

This study aimed to investigate the role of sestrin2 in cancer stemness of CRC. Firstly, the effects of up-regulating Sestrin2 on the survival, migration, and colony formation of CRC cells were studied and the expression of cancer stemness in high-expression Sestrin2 CRC cells were detected. Secondly, the relationship between Sestrin2 and Wnt signalling was explored. Finally, we observed that up-regulation of Sestrin2 inhibited tumor growth in CRC cells *in vivo*. Our results suggest that Sestrin2 inhibits CRC cell progression by downregulating the Wnt signalling pathway.

Materials And Methods

Cell culture

The human CRC cell lines were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). HCT-116 were cultured in McCoy's 5A medium (Biological Industries, Beit HaEmek, Israel) and SW620 cells were cultured in RPMI 1640 medium (Biological Industries) supplemented with 10% fetal bovine serum (FBS) (Biological Industries). Cells were cultured in 37°C with 5% of CO2 concentrations in incubators.

Infection with lentiviral con°Cstructs

Lentiviral constructs expressing Sestrin2 (LV-Sestrin2) and a non-targeted green fluorescence protein (GFP) virus (LV-GFP) were purchased from GeneChem (Shanghai, China). Cells ($1.5´10^4$) were plated in 24-well plates on the day before transfection. Lentiviral constructs were transduced at multiplicity of infection 10 in HCT-116 and SW620 cells using HiTransG P transfection reagent (Genechem). The infected cells were maintained in fresh medium without FBS for 12 h and then washed with phosphate-buffered saline (PBS), after which the medium was replaced with fresh medium supplemented with 10% FBS. Puromycin (2 mg/mL; Beyotime, Beijing, China) was used to generate stable expression cell lines in HCT116 and SW620 cells.

Quantitative PCR

The RNAsimple Total RNA Kit (TIANGEN, Beijing, China) was used to extract total RNA according to the manufacturer's instructions. All-in-One cDNA Synthesis SuperMix (Bimake, USA) was used to generate the

first-strand cDNA. The expression level of cDNAs was normalized to that of β-actin by the comparative CT method. The primer sequences used in this study are provided in Table 1.

Cell proliferation assay

A total of 500 cells were plated in the wells of a 96-well plate. Cell viability was measured using Cell Counting Kit-8 (CCK-8) (Bimake) and the Thermo Scientific™ Varioskan™ LUX Multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm once a day for 7 days.

Sphere formation

Sphere cells were cultured in stem cell medium (SCM), which is a DMEM/F-12 medium supplemented with epidermal growth factor (20 ng/mL, MedChemExpress, MCE), basic fibroblast growth factor (20 ng/mL, MCE), and B-27 (2%; Invitrogen, Carlsbad, CA, USA). A total of 200 cells were plated into each 96-well with 200 μL SCM. The number of spheres was counted after 2 weeks. For second sphere assay, the sphere cells were separated using ACCUTASE (YEASEN). A total of 200 cells were plated into each 96-well with 200 μL SCM. The number of spheres was counted after 2 weeks.

Limiting dilution assay in vitro

The procedure is same as described before [20]. Briefly, cells were seeded in U-bottom 96-well at final cell concentrations 1000, 100, 10, 1 per well with 200 μL SCM. After 2 weeks, the wells contain cell spheres were counted and empty wells were excluded. The frequency of CSCs was calculated on website (http://bioinf.wehi.edu.au/software/elda/) [21].

Transwell assay

Total 1×10^6 suspended cells were added in the upper chamber (0.8 um in 6 well) with serum-free culture medium of transwell chamber (JET, China). The lower chamber contained medium supplemented with 10% FBS. After 24 h cultured, cells on the upper side of the filter membrane were removed. The rest of the cells were fixed with 5% paraformaldehyde for 20 min and dyed using Crystal Violet Staining Solution (Beyotime, China), then photographed at 200× and counted in five random fields.

Flow cytometry

Cells were detached into single cells and washed with cold PBS, pre-incubated with Human TruStain FcX™ (422301; BioLegend, San Diego, CA, USA), and then incubated with CD44-APC (B265921; BioLegend) on ice in the dark. Cells were assayed by flow cytometry.

Soft agar colony formation assay

A concentration of 1×10^4 cells was suspended in SCM containing 0.36% agar and poured on an agar bed (SCM with 0.75% agar). After 3 weeks, the sphere number was counted after 0.04% crystal violet staining.

Wnt/β-catenin pathway inhibition assay

HCT-116 cells in the LV-Sestrin2 and LV-GFP groups were treated with 0.5 μM BML-284 (Wnt signaling activator; MedChemExpress, Monmouth Junction, NJ, USA) in dimethyl sulfoxide for 24 h and then collected for Western blot analysis. In the sphere formation assay, 0.1 μM BML-284 was mixed with cells and then plated in 96 wells with SCM. The number of spheres was counted after 2 weeks.

Western blot analysis

Cells were washed with PBS and lysed in RIPA buffer with Protease Inhibitor Cocktail (Bimake) on ice. Cell lysates were centrifuged (12,000 rpm) at 4°C for 20 min and then quantified using the BCA Protein Assay Kit (Beyotime). The lysate was denatured with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Sample Loading Buffer (Beyotime), followed by SDS-PAGE and electrotransfer to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were incubated overnight at 4°C with anti-Sestrin2, anti-CD44, anti-Sox2, anti-Oct4, anti-CXC chemokine receptor 4 (Cxcr4), and anti-β-actin, (Proteintech Group, Wuhan, China) at the appropriate dilution, and incubated with secondary antibody at room temperature for 2 h. The bands were visualized using BeyoECL Plus (Beyotime).

Xenograft mouse model

A xenograft mouse model was determined by subcutaneously injection. One million HCT-116 cells in the LV-Sestrin2 and LV-GFP groups injected into the hips of 6-week-old female BALB/c-nu mice (Animal Center of Chongqing Medical University, Chongqing, China). All animal studies were approved by the Ethics Committee of Chongqing Medical University. The tumour size was measured every 3 days. The tumour volume (V= l×w2/2) was calculated by measuring the length (l) and width (w). Mice were euthanized by increasing volume of CO_2in a chamber, 21 days after cells injection.

Bioinformatics Analysis

The Colorectal cancer dataset used comprised mRNA-seq data from TCGA tumors (https://tcga-data.nci.nih.gov/tcga/). The two-gene correlation map is realized by the R software package ggstatsplot, and the multi-gene correlation map is displayed by the R software package pheatmap. We used Spearman's correlation analysis to describe the correlation between quantitative variables without a normal distribution. A p-value of less than 0.05 was considered statistically significant.

Statistical analysis

Except where otherwise noted, experiments were repeated at least three times. All statistical analyses were performed using GraphPad Prism Version 8. The t test was used to evaluate the significance of the differences between two groups of data. P <0.05 was considered statistically significant.

Results

Sestrin2 inhibits CRC cell proliferation, migration, and colony formation

To evaluate the effects of Sestrin2 on CRC, we firstly investigated the mRNA expression of Sestrin2 compared to corresponding adjacent tissues in The Cancer Genome Atlas datasets using the UALCAN website (UALCAN: http://ualcan.path.uab.edu) (Fig. 1A) [22]. The results showed that Sestrin2 was significantly downregulated in both subtypes of CRC, which are colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ). Secondly, we introduced LV-Sestrin2 and LV-GFP into HCT116 and SW620 cells, and proliferation, migration, and colony formation were evaluated. The CCK-8 assay showed that Sestrin2 inhibited the proliferation of CRC cells (Fig. 1B). The results from our colony formation assay demonstrated that the number of colonies formed was decreased in LV-Sestrin2-transduced CRC cells (Fig. 1C). Furthermore, the transwell showed that the migration of LV-Sestrin2-transduced CRC cells was inhibited (Fig. 1D) and wound healing assay also showed a consistent result (Fig. 1E). These results demonstrate that Sestrin2 is downregulated in CRC patients and has anti-cancer effects on CRC cells.

Sestrin2 Inhibits The Cancer Stemness In Crc Cells

CSCs play a significant role in cancer progression such as tumour growth, recurrence, and metastasis [23]. Therefore, we hypothesized Sestrin2 effects on CSCs in CRC cells. Self-renew and initiating tumor ability are basic characteristics of CSCs[24]. Soft agar assay is able to imitate vivo micro-environment[25]. We used the sphere assay and soft agar colony formation assay to investigate the self-renew ability of CRC cells. The results showed that Sestrin2 suppressed tumoursphere formation (Fig. 2A). Moreover, to determine the colorectal cancer initiating cells, we used ELDA assay and the results showed that LV-Sestrin2 reduced the CSCs in HCT-116 and SW620 cell lines (Table 1). In addition, molecules relating to CSCs were evaluated. Flow cytometry showed that the percentage of CD44 + cells was decreased in Sestrin2-transfected CRC cells (Fig. 2B). Expectedly, the stemness-related markers, including CD44, Oct4, Sox2, and Cxcr4, was downregulated at both the mRNA and protein levels in HCT-116 and SW620 cells (Fig. 2C-D) and similar protein expression trends can also be observed in sphere cells (**Supplement** Fig. 1). These results indicate the significant effects of Sestrin2 on reducing the cancer stemness in CRC cells.

Sestrin2 Reduces The Cancer Stemness Via The Wnt/β-catenin Pathway

As the Wnt/β-catenin pathway is one pathway that regulates stemness [26]. Activated Wnt/β-catenin pathway increases the number of CSCs by regulating self-renewal and homeostasis in cancer cell[27] .We observed the protein expression of β-catenin and the downstream target c-Myc. Our observations showed the expression of β-catenin and c-Myc was downregulated (Fig. 3A). Furthermore, we used the Wnt/β-catenin pathway activator, BML-284, to rescue the effects of Sestrin2 and found that BML-284 was able

to partly rescue the effects of Sestrin2 using the sphere assay (Fig. 3B-C), consistent with the Western blot analyses in HCT-116 cells (Fig. 3D). In addition, to investigate whether sestrin2 affect cancer stemness by regulating Reactive Oxygen Species (ROS), Scavengers of ROS, Sodium pyruvate and Carboxy-PTIO, were used and we found Scavengers of ROS not affect the protein expression of CD44, no matter a single use or a joint use (**Supplement** Fig. 2). Our findings suggest that Sestrin2 regulates the cancer stemness via the Wnt/β-catenin pathway.

Sestrin2 Inhibits The Cancer Stmness In Vivo

To evaluate the effects of Sestrin2 *in vivo*, a subcutaneous xenotransplant tumour model was used. A concentration of 1×10^7 cells from the LV-Sestrin2 or LV-GFP groups was injected into each of three female BALB/c-nu mice. The volume of tumour produced by HCT116-Sestrin2 cells was significantly reduced compared with that from HCT116-GFP cells when the weight of mice was not significantly different (Fig. 4A-E). Western blot analysis showed that the upregulation of Sestrin2 inhibited the protein levels of Sox2, c-Myc, and β-catenin (Fig. 4F). Further, in order to confer our finding in CRC patients, the mRNA expression correlations between Sestrin2 and CD44, Oct4, Sox2, Cxcr4, c-Myc and β-catenin were detected by R using TCGA data. The mRNA expression of CD44, β-catenin, Cxcr4 and c-Myc were negatively related to Sestrin2, which consistent with our results *in vitro* (Fig. 4G). The efficiency of tumour formation and cancer stemness was decreased by Sestrin2 *in vitro*.

Discussion

Previous studies have introduced the role of Sestrin2 in CRC. In this study, we further investigated the role of Sestrin2 in CRC and found Sestrin2 inhibits the cancer stemness both *in vivo* and *in vitro*. Using primary and secondary sphere formation assay to detect the self-renew of CRCs, we found Sestrin2 up-regulating inhibited sphere formation of CRC cells. Consistently, The ELDA suggested that Sestrin2 inhibited the initial ability. Further, the expression of CD44, a key marker for CRC, and Oct4, Sox2, and Cxcr4, the stem cell factors, was also decreased. Overall, our data suggested that Sestrin2 not only reduce self-renew but also the initial ability of CRCs.

Sestrin2 may be a therapeutic target. First of all, CSC plays a very important role in cancer including maintenance, self-renewal, division, and tumour development [28]. As tumour propagation initiators, CSCs are considered to be a promising therapeutic method [29]. Besides, in this study, Sestrin2 targeting the Wnt/β-catenin pathway reduced the cancer stemness of CRC cells and Sestrin2 inhibited the proliferation, migration, and colony formation of CRC cells. If a medicine targeting on Sestrin2 would affect some tumor biological characteristics of CRC. Thus, Sestin2 may be a promising therapeutic target for CRC.

It is wildly accepted that the epithelial-mesenchymal transition (EMT) and CSCs are two closely related processes in tumour progression and therapeutic resistance [30]. In studies form other teams, Sestrin2

was shown to inhibit the EMT [31, 32]. These results can become evidence for our results which is Sestrin2 regulating the cancer stemness.

Sestrin2 is an anti-cancer molecule. In CRC, the decreased expression of Sestrin2 in patients predicts unfavorable outcomes, and Sestrin2 is also an important facilitator of the p53-mediated control of cancer cell growth [17, 33]. Moreover, Sestrin2 has been shown to function as a tumour suppressor in lung cancer, hepatocellular carcinoma, and melanoma [34–36]. Anti-tumour molecules such as tanshinone IIA, fangchinoline, and nelfinavir work together with Sestrin2 to carry out these functions [37–39]. This study also showed that Sestrin2 also has anti-cancer effects.

The effects of Sestrin2 on Wnt pathway may be via AKT signal pathway. Usually, Sestrin2, known as a p53 target gene, inhibits mTORC1 signaling and induction of autophagy [40, 41]. Interestingly, our results indicated that Sestrin2 also downregulates the Wnt/β-catenin signaling pathway. The reason why our results differ from others may be that SENS2 activates AKT and AMPK signaling [34] and PI3K/Akt/Wnt/β-catenin signaling, which controls the levels of EMT-related proteins [32]. Thus, Sestrin2 may affect β-catenin by interacting with AKT.

In conclusion, the results of this study showed that LV-Sesrin2 inhibits cancer stemness through the Wnt pathway in HCT-116 and SW620 cells, suggesting that Sesrin2 may be a promising therapeutic target for CRC.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the first affiliated hospital of chongqing medical university.

Consent for publication

All authors consent for publication.

Competing interests

The authors declare that no competing interests exist.

Funding

This study was funded by the Chongqing Science and Technology Commission (No. cstc2017jcyjAX0127).

Author contributions

JW and ZF designed the experiments. ZF supervised the whole project. XZ performed the major research and wrote the paper. WL and XL provide their professional guidance. XZ and WL provided the technical support.

Acknowledgments

All authors thank professor Weiyin Zhou from the College of Pharmacy, Chongqing Medical University, for research equipment and support.

Availability of data and materials

The data and materials can be obtained from the first author and corresponding author.

References

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. CA Cancer J Clin. 2016. 66(2): 115-32.
2. van der Heijden M, Vermeulen L. Stem cells in homeostasis and cancer of the gut. Mol Cancer. 2019. 18(1): 66.
3. Plaks V, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells. *Cell Stem Cell*. 2015;16(3):225-38.
4. Karsten U, Goletz S. What makes cancer stem cell markers different. *Springerplus*. 2013;2(1):301.
5. Tomao F, Papa A, Rossi L, et al. Emerging role of cancer stem cells in the biology and treatment of ovarian cancer: basic knowledge and therapeutic possibilities for an innovative approach. *J Exp Clin Cancer Res*. 2013;32:48.
6. Deshmukh A, Deshpande K, Arfuso F, Newsholme P, Dharmarajan A. Cancer stem cell metabolism: a potential target for cancer therapy. *Mol Cancer*. 2016;15(1):69.
7. Vlashi E, Pajonk F. Cancer stem cells, cancer cell plasticity and radiation therapy. *Semin Cancer Biol*. 2015;31:28-35.
8. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene*. 2017;36(11):1461-1473.
9. Espada J, Calvo MB, Díaz-Prado S, Medina V. Wnt signalling and cancer stem cells. *Clin Transl Oncol*. 2009;11(7):411-27.
10. Koveitypour Z, Panahi F, Vakilian M, et al. Signaling pathways involved in colorectal cancer progression. *Cell Biosci*. 2019;9:97.
11. Park HW, Park H, Ro SH, et al. Hepatoprotective role of Sestrin2 against chronic ER stress. Nat Commun. 2014. 5: 4233.

12. Budanov AV. Stress-responsive sestrins link p53 with redox regulation and mammalian target of rapamycin signaling. *Antioxid Redox Signal*. (2011) 15:1679-90. doi:10.1089/ars.2010.3530
13. Lee JH, Budanov AV, Karin M. Sestrins orchestrate cellular metabolism to attenuate aging. *Cell Metab*. (2013) 18:792-801. doi:10.1016/j.cmet.2013.08.018
14. Pasha M, Eid AH, Eid AA, Gorin Y, Munusamy S. Sestrin2 as a Novel Biomarker and Therapeutic Target for Various Diseases. *Oxid Med Cell Longev*. (2017) 2017:3296294. doi:10.1155/2017/3296294
15. Chae HS, Gil M, Saha SK, Kwak HJ, Park HW, Vellingiri B, et al. Sestrin2 Expression Has Regulatory Properties and Prognostic Value in Lung Cancer. *J Pers Med*. (2020) 10. doi:10.3390/jpm10030109
16. Shin J, Bae J, Park S, Kang HG, Shin SM, Won G, et al. mTOR-Dependent Role of Sestrin2 in Regulating Tumor Progression of Human Endometrial Cancer. *Cancers (Basel)*. (2020) 12. doi:10.3390/cancers12092515
17. Wei JL, Fu ZX, Fang M, et al. Decreased expression of sestrin 2 predicts unfavorable outcome in colorectal cancer. Oncol Rep. 2015. 33(3): 1349-57.
18. Kim GT, Lee SH, Kim JI, Kim YM. Quercetin regulates the sestrin 2-AMPK-p38 MAPK signaling pathway and induces apoptosis by increasing the generation of intracellular ROS in a p53-independent manner. Int J Mol Med. 2014. 33(4): 863-9.
19. Seo K, Seo S, Ki SH, Shin SM. Sestrin2 inhibits hypoxia-inducible factor-1α accumulation via AMPK-mediated prolyl hydroxylase regulation. Free Radic Biol Med. 2016. 101: 511-523.
20. Agro L, O'Brien C. In vitro and in vivo Limiting Dilution Assay for Colorectal Cancer. Bio Protoc. 2015;5:1-11.
21. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347:70-8.
22. Chandrashekar DS, Bashel B, Balasubramanya S, et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. Neoplasia. 2017. 19(8): 649-658.
23. Chang JC. Cancer stem cells: Role in tumor growth, recurrence, metastasis, and treatment resistance. Medicine (Baltimore). 2016. 95(1 Suppl 1): S20-5.
24. Li Y, Liu YH, Hu YY, Chen L, Li JM. Special AT-rich sequence-binding protein 2 acts as a negative regulator of stemness in colorectal cancer cells. *World J Gastroenterol*. (2016) 22:8528-39. doi:10.3748/wjg.v22.i38.8528
25. Horibata S, Vo TV, Subramanian V, Thompson PR, Coonrod SA. Utilization of the Soft Agar Colony Formation Assay to Identify Inhibitors of Tumorigenicity in Breast Cancer Cells. *J Vis Exp*. (2015) :e52727. doi:10.3791/52727
26. Takebe N, Miele L, Harris PJ, et al. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol. 2015. 12(8): 445-64.
27. Najafı M, Farhood B, Mortezaee K. Cancer stem cells (CSCs) in cancer progression and therapy. J Cell Physiol. 2019. 234(6): 8381-8395.

28. Jariyal H, Gupta C, Bhat VS, Wagh JR, Srivastava A. Advancements in Cancer Stem Cell Isolation and Characterization. Stem Cell Rev Rep. 2019. 15(6): 755-773.
29. Eun K, Ham SW, Kim H. Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting. BMB Rep. 2017. 50(3): 117-125.
30. Liu X, Fan D. The epithelial-mesenchymal transition and cancer stem cells: functional and mechanistic links. Curr Pharm Des. 2015. 21(10): 1279-91.
31. Jia Y, Zheng Z, Yang Y, et al. MiR-4756 promotes albumin-induced renal tubular epithelial cell epithelial-to-mesenchymal transition and endoplasmic reticulum stress via targeting Sestrin2. J Cell Physiol. 2019. 234(3): 2905-2915.
32. Lee K, Chang JW, Oh C, et al. HOXB5 acts as an oncogenic driver in head and neck squamous cell carcinoma via EGFR/Akt/Wnt/β-catenin signaling axis. Eur J Surg Oncol. 2019 .
33. Ro SH, Xue X, Ramakrishnan SK, et al. Tumor suppressive role of sestrin2 during colitis and colon carcinogenesis. Elife. 2016. 5: e12204.
34. Dai J, Huang Q, 0000-0002-1071-1696 AO, et al. Sestrin 2 confers primary resistance to sorafenib by simultaneously activating AKT and AMPK in hepatocellular carcinoma. Cancer Med. 2018. 7(11): 5691-5703.
35. Xu H, Sun H, Zhang H, et al. An ShRNA Based Genetic Screen Identified Sesn2 as a Potential Tumor Suppressor in Lung Cancer via Suppression of Akt-mTOR-p70S6K Signaling. PLoS One. 2015. 10(5): e0124033.
36. Zhao B, Shah P, Budanov AV, et al. Sestrin2 protein positively regulates AKT enzyme signaling and survival in human squamous cell carcinoma and melanoma cells. J Biol Chem. 2014. 289(52): 35806-14.
37. Bruning A, Rahmeh M, Friese K. Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. Mol Oncol. 2013. 7(6): 1012-8.
38. Wang N, Pan W, Zhu M, et al. Fangchinoline induces autophagic cell death via p53/sestrin2/AMPK signalling in human hepatocellular carcinoma cells. Br J Pharmacol. 2011. 164(2b): 731-42.
39. Yen JH, Huang ST, Huang HS, et al. HGK-sestrin 2 signaling-mediated autophagy contributes to antitumor efficacy of Tanshinone IIA in human osteosarcoma cells. Cell Death Dis. 2018. 9(10): 1003.
40. Liang Y, Zhu J, Huang H, et al. SESN2/sestrin 2 induction-mediated autophagy and inhibitory effect of isorhapontigenin (ISO) on human bladder cancers. Autophagy. 2016. 12(8): 1229-39.
41. Wolfson RL, Chantranupong L, Saxton RA, et al. Sestrin2 is a leucine sensor for the mTORC1 pathway. Science. 2016. 351(6268): 43-8.

Tables

TABLE 1

Limiting dilution data showing the effect of Sestrin2 expression on the frequency of CSCs

Cells seeded		1	10	100	1000	Estimated Frequency of CSCs	p value
HCT-116	LV-GFP	62/78	152/162	89/92	24/24	1 in 6 cells	0.087
	LV-Sstrin2	78/105	136/170	86/91	24/24	1 in 7 cells	
SW620	LV-GFP	45/90	101/145	76/94	24/24	1 in 19 cells	4.24×10-8
	LV-Sstrin2	51/128	96/172	74/96	22/24	1 in 35 cells	

Figures

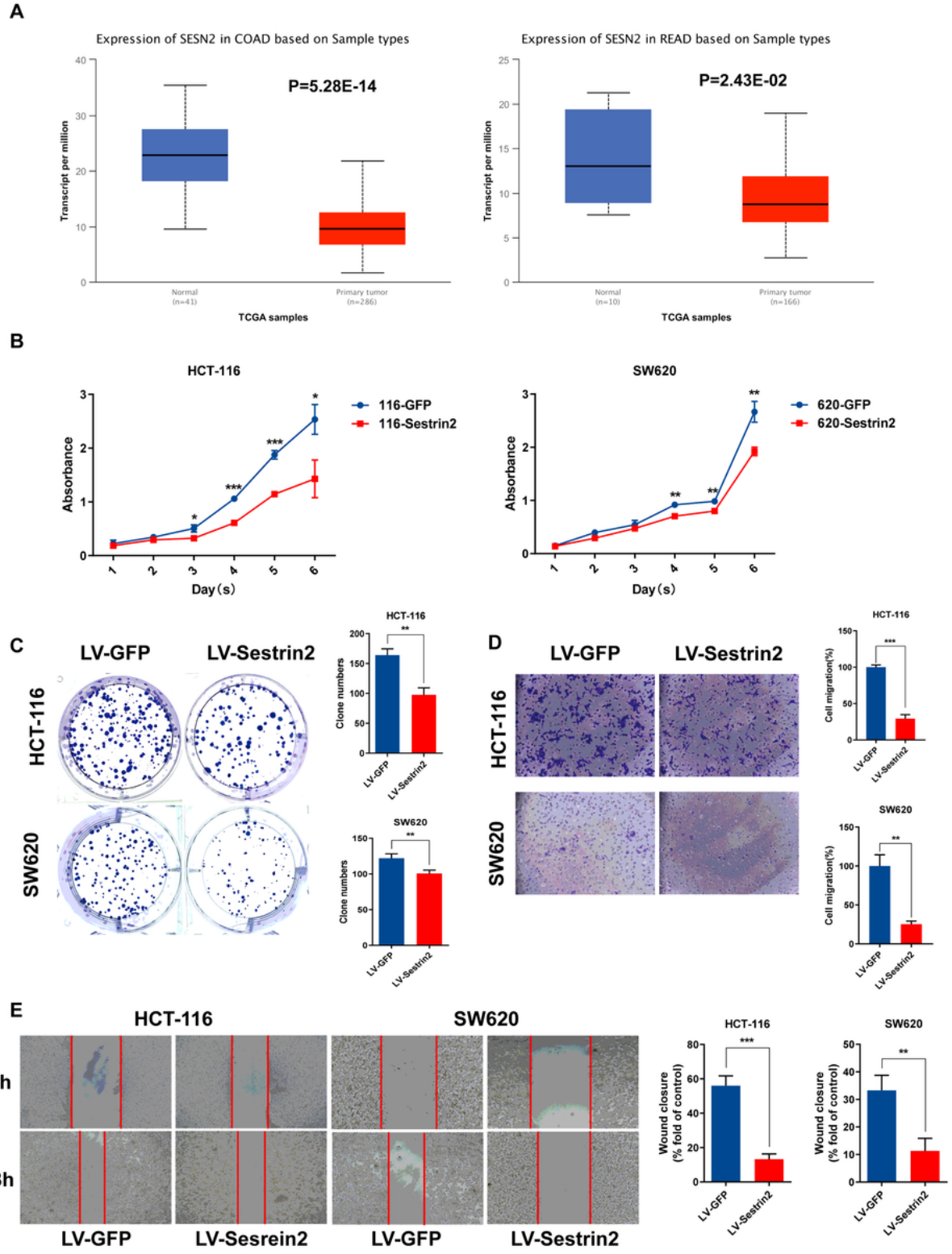

Figure 1

Sestrin2 has anti-cancer effects on CRC cells. (A) Sestrin2 was down-regulated in both COAD (P = 0.28×10-14) and READ (P = 0.024) compared to normal tissue in TCGA. (B) Cell proliferation was markedly inhibited after Sestrin2 up-regulation as determined by the CCK-8 assay. (C) The colony formation assay showed that the number of colonies formed by LV-Sestrin2-infected cells was smaller than that of LV-GFP-infected cells; ** P < 0.01. The right panel shows the number of colonies. (D) Cell

migration was detected by transwell assay. The cell number was counted by Imagj. (E) Cell migration was measured by the wound healing assay. The area of migration was quantified as the mean ± standard deviation (SD) of the area normalized to the respective LV-GFP group (right panel); *** P < 0.001.

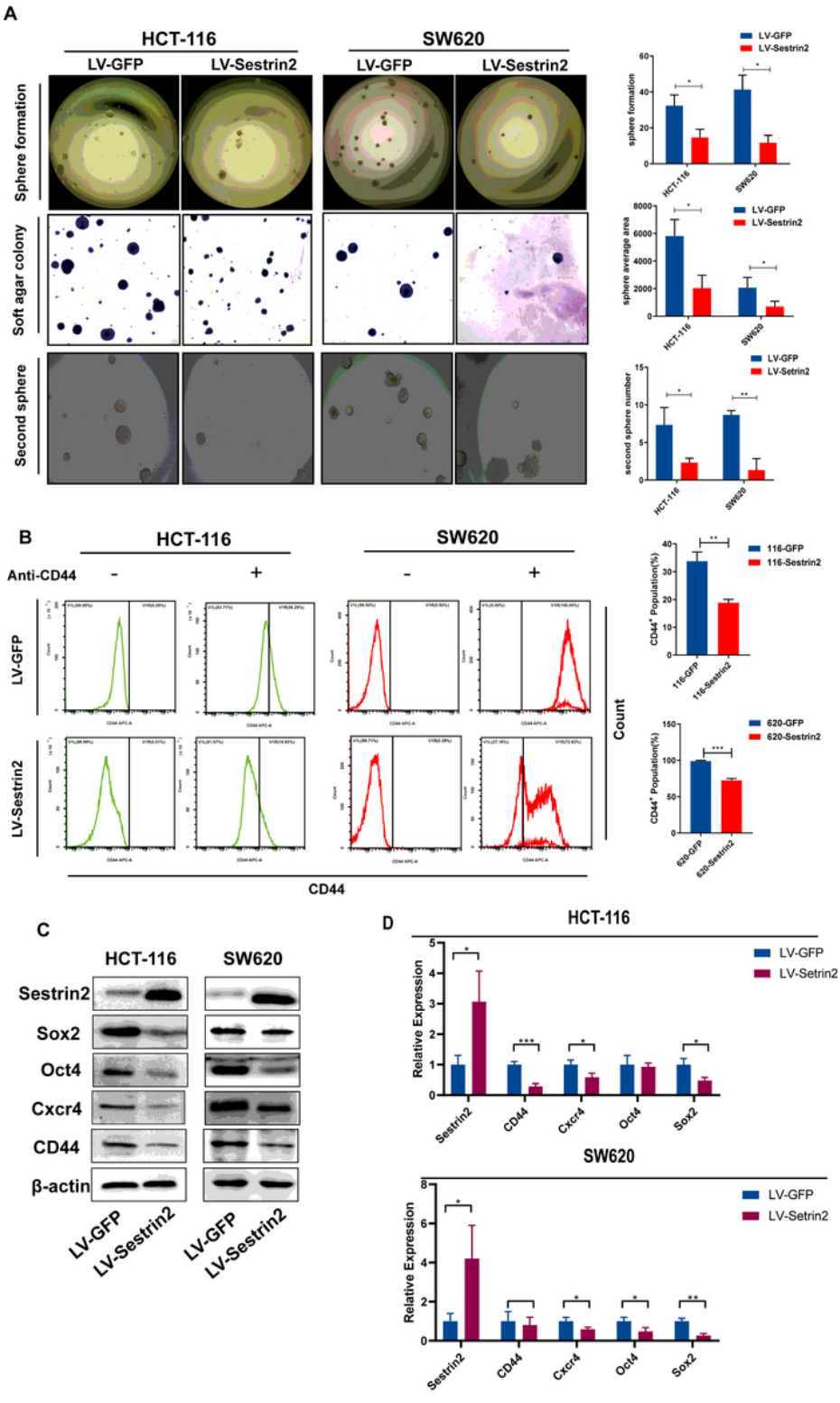

Figure 2

Sestrin2 inhibits the cancer stemness in CRC cells. (A) The cell sphere formation assay (up), soft agar colony formation assay (meddle) and Second cell sphere formation (down) showed that the number of

spheres and the average area of soft agar colony were both decreased; * P < 0.05 ** P < 0.01. (B) The percentages of CD44+ cells in the LV-GFP group were larger than those in the LV-Sestrin2 group. The (C) Representative Western blot images of the effect of LV-Sestrin2 on the expression levels of Sestrin2, Sox2, Oct4, Cxcr4, and CD44. β-actin was used as a loading control. (D) The relative mRNA expression of Sestrin2, Sox2, Oct4, Cxcr4, and CD44 in LV-Sestrin2 CRC cells normalized to the LV-GFP group.

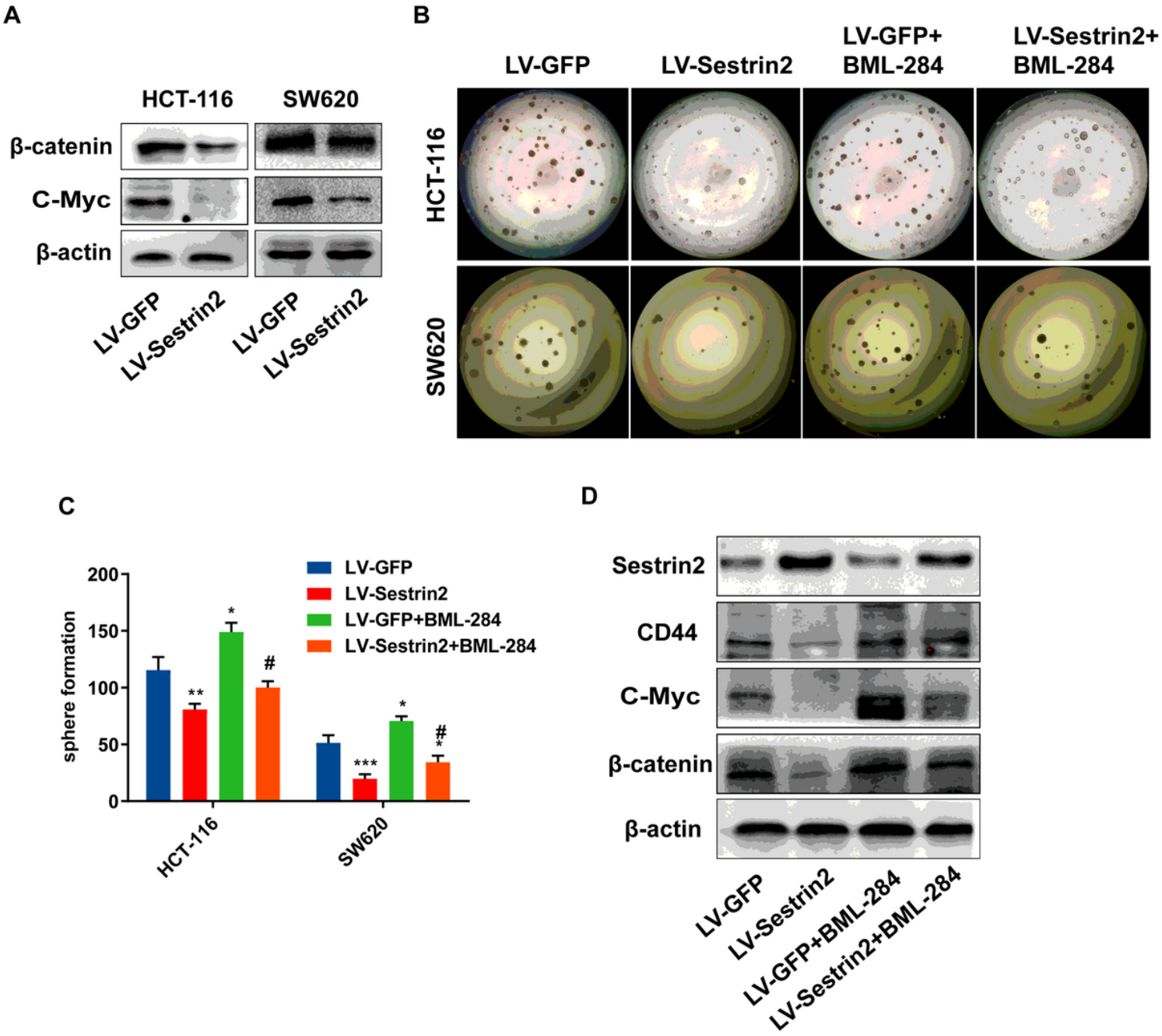

Figure 3

The effects of Sestrin2 on the cancer stemness are mediated by the Wnt/β-catenin pathway. (A) Western blot analysis of Wnt/β-catenin signaling-related proteins. (B) The sphere formation assay in LV-Sestrin2 and LV-GFP CRC cells with or without exposure to BML-284 (0.1 μM). (C) Number of spheres; * P < 0.05 compared with LV-GFP, #P < 0.05 compared with LV-Sestrin2. (D) Western blot analysis of proteins related

to the Wnt/β-catenin pathway (β-catenin, c-Myc), cancer stemness (CD44) in LV-Sestrin2 and LV-GFP CRC cells with or without incubation with BML-284 (0.5 μM) for 24 h in HCT-116 cells.

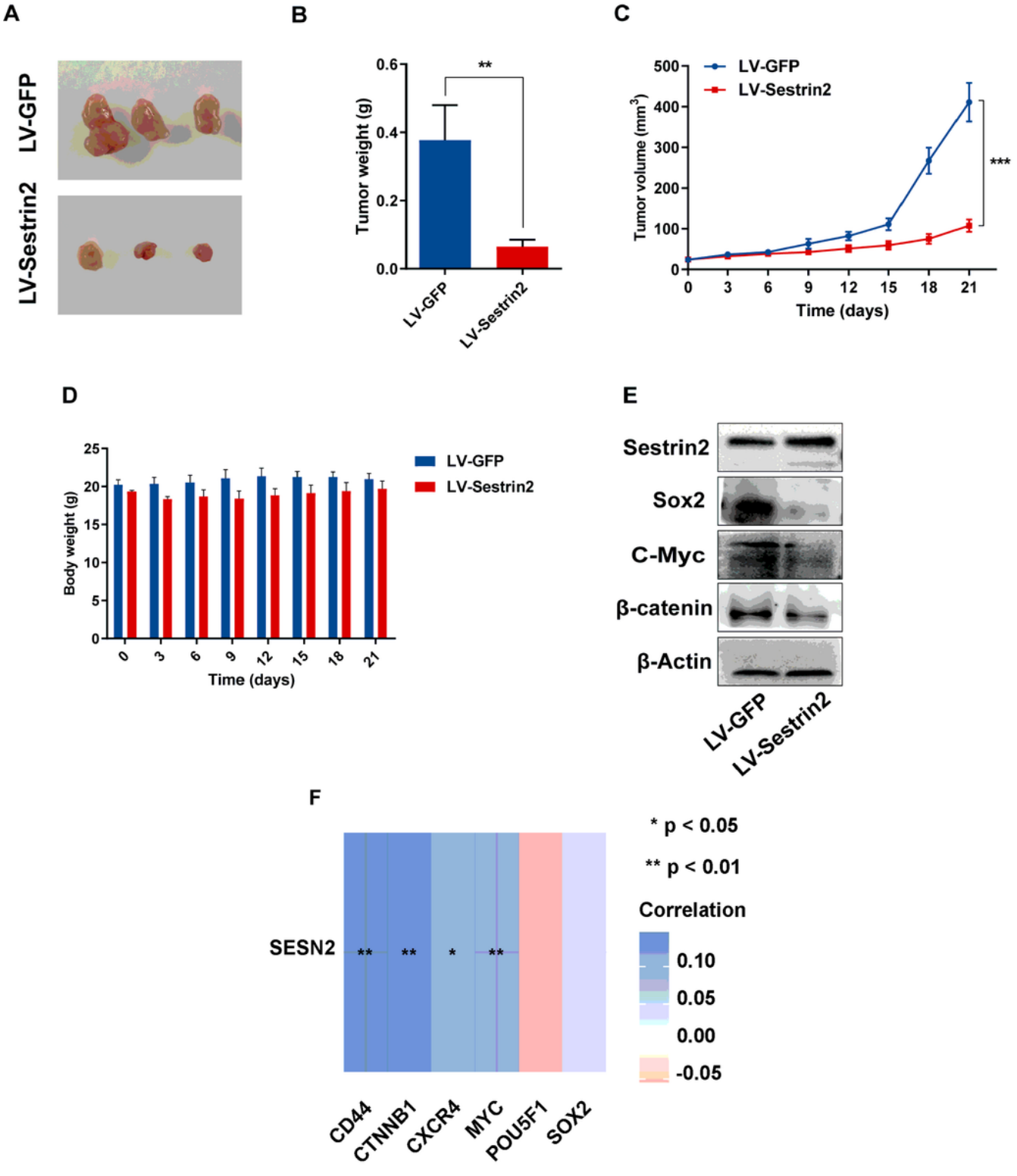

Figure 4

Sestrin2 inhibits cancer stemness in vivo. (A) LV-Sestrin2 and LV-GFP cells were transplanted into the hips of mice for 3 weeks, (B) and then the tumours were excised. (C) Tumour volume data. (D) T The tumour weights were measured. (E) Mice body weights. (F) Western blots of Sestrin2, Sox2, c-Myc, and β-

catenin. (G) A heat map of the correlation between CD44, β-catenin (CTNNB1), CXCR4, c-Myc (MYC), OCT4 (POU5F1), SOX2 and Sestrin2 (SESN2). Red represents positive correlation and blue represents negative correlation, and the darker the color represents the two stronger correlations. Asterisks represent levels of significance (*p < 0.05, **p < 0.01).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementTable1.docx
- supplimentfigure1.tif
- supplimentfigure2.tif