ROS

RESEARCH ARTICLE

Elimination of Sestrin 2 Compromises Viability in Extracellular Matrix-Detached SKOV3 Ovarian Cancer Cells

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ABSTRACT | Epithelial ovarian carcinoma (EOC) is considered the deadliest gynecological cancer, largely due to the fact that it is often diagnosed once the cancer has already metastasized, thus making the disease more difficult to treat. Throughout metastasis, ovarian epithelial cancer cells must overcome many feats, including surviving in extracellular matrix (ECM) detachment. ECM-detached cancer cells must evade a number of insults, including increased intracellular reactive oxygen species (ROS). Recent evidence suggests ECM-detached cancer cells rely on antioxidant enzymes to combat these increasing levels of ROS to promote survival; however, the specific antioxidant enzymes involved in this process have yet to be fully elucidated. Sestrin 2 (SESN2) is a multi-functional protein that has been found to be instrumental in many different signaling pathways; notably, it has been recognized to play a critical role in eliminating ROS. Here, we show that SESN2 plays a unique role in maintaining the viability of ECM-detached metastatic ovarian epithelial cancer cells, and elimination of this critical protein results in compromised viability. Thus, these data identify SESN2 as a potentially interesting therapeutic target for treating this deadly metastatic disease.

KEYWORDS | Extracellular matrix; Ovarian cancer; Reactive oxygen species; Sestrin

ABBREVIATIONS | ECM, extracellular matrix; EOC, epithelial ovarian carcinoma; ROS, reactive oxygen species; SESN2, sestrin 2; shRNA, short hairpin RNA

CONTENTS

1. INTRODUCTION

Epithelial ovarian carcinoma (EOC) is the most common form of ovarian cancer and is often more aggressive than nonepithelial malignancies [1]. Given their aggressive nature, 65% of EOC cases are diagnosed after the disease has already metastasized (or spread) to other areas throughout the abdominal cavity or to distant organs, thus making the disease more difficult to treat [1]. It has become clear that better therapeutic options are needed to remedy patients that present with this disease. During metastasis, EOC cells escape the ovaries and use the peritoneal fluid to travel to organs throughout the abdominal cavity before eventually utilizing the bloodstream and lymphatics to travel to distant areas, including the skin, lungs, and brain [2]. Throughout this process, EOC cells must gain the ability to thrive while being detached from the extracellular matrix (ECM) [3–5]. It has been recognized that ECM-detached breast cancer cells must overcome many ECM detachment-induced insults, including increases in reactive oxygen species (ROS) [3, 4]. Specifically, ECM-detached breast cancer cells combat increases in ROS through utilizing antioxidant enzymes, a family of enzymes that facilitate oxidant scavenging [3]. However, the importance of antioxidant enzymes in the successful metastasis of EOC cells has yet to be fully elucidated.

Sestrin 2 (SESN2) is a member of a family of proteins (SESN1-3) that are highly conserved and expressed in humans [6]. While this stress-inducible protein appears to lack intrinsic catalytic antioxidant activity, SESN2 is involved in processes that decrease ROS levels [7]. Specifically, SESN2 promotes oxidoreductase activity and mediates the expression of Nrf2, a transcription factor responsible for controlling antioxidant enzyme expression [7, 8]. Most recently, SESN2 has been found to be implicated in cancer. Decreased SESN2 expression in colorectal cancer tissues has been connected with poor prognosis [9]. Furthermore, increased SESN2 expression has been connected with successful ionizing radiation-inducing killing of breast cancer cells [10]. While recent studies have begun to unravel the role of SESN2 in tumorigenesis, SESN2 and its importance in EOC, specifically its importance in anchorage independence and metastasis, has yet to be explored.

Here, we sought to determine the role of SESN2 in the viability and proliferation of ECM-detached SKOV3 cells, an EOC cell line. We discovered that SESN2 uniquely protects the viability of anchorage-independent SKOV3 cells, and elimination of SESN2 in ECM-detached SKOV3 cells results in substantial cell death. In aggregate, these data define SESN2 as a new potential therapeutic target for metastatic EOC.

2. MATERIALS AND METHODS

2.1. Cell Culture

SKOV3 ovarian cancer cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in McCoy's 5A media modified with L-glutamine and sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS) (Alkali Scientific, Ocala, FL, USA), and 1% penicillin/streptomycin (Gibco, Waltham, MA, USA). The cells were maintained at 37°C and 5% CO_2.

2.2. Short Hairpin RNA (shRNA)

MISSION shRNA targeting SESN2 was purchased from Sigma-Aldrich, and stable knockdown of SESN2 was achieved using lentiviral transduction as described previously [11].

2.3. Western Blotting

Western blotting was performed as described previously [11]. To confirm SESN2 knockdown, SKOV3 parental and SKOV3 SESN2 cells were lysed in 1%

NP-40 (Sigma-Aldrich) with aprotinin (1 µg/ml) (Gibco) and leupeptin (5 µg/ml) (Gibco) for 20 min, spun at 18,188 *g* for 30 min at 4ºC, and normalized using a BCA assay (Pierce Biotech, Rockford, IL, USA). Laemmeli loading dye (Bio-Rad, Hercules, CA, USA) was added to normalized lysates and lysates were run at 150 V on a 12% SDS-PAGE gel (Bio-Rad) before being transferred to PVDF membrane (Millipore, St. Louis, MO, USA) at 100 V. The membrane was blocked in 5% milk in 1× TBST (Bio-Rad) for 30 min and incubated with the primary antibodies used at a dilution of 1:1000 for rabbit anti-SESN2 (cat # 10795-1-AP, Proteintech, Rosemont, IL, USA) and mouse anti-β actin (cat # 66009-1-Ig, Proteintech) as a loading control overnight. The blot was washed with 1× TBST (Bio-Rad) three times for 20 min before the goat anti-rabbit HRP-conjugated (cat # SA00001-2, Proteintech) and goat anti-mouse HRP-conjugated (cat # SA00001-1, Proteintech) secondary antibodies were used at a dilution of 1:1500 in 5% milk and 1× TBST. The membrane was washed three times for 10 minutes using 1× TBST before being developed using chemiluminescence (Alkali Scientific).

2.4. Soft Agar Assay

The soft agar assay was completed as described previously [11]. Briefly, a mixture of 0.4% agarose and media (2 ml) were poured into each well of a six-well plate. SKOV3 parental cells or SKOV3 SESN2-deficient cells were plated in triplicate on the six-well plate at 20,000 cells per well in 1.5 ml of 0.5% agarose (Sigma-Aldrich) and were plated on top of cooled 0.4% media and agarose wells. Plates were incubated for 21 days at 37ºC and 5% CO_2 and wells were fed with 1 ml of media every two days. This experiment was repeated at least three times, and colonies were counted per well using ImageJ software (NIH, Bethesda, MD, USA).

2.5. Cell Proliferation and Cell Viability Assay

Cell proliferation and viability in ECM attachment were determined by using trypan blue exclusion as described before [11]. Approximately 50,000 cells were plated in 1 ml of media per well in triplicate for SKOV3 parental and SKOV3 SESN2-deficient cells in ECM attachment using six-well tissue culture plates (Cytone 1, Ocala, Florida, USA) or in ECM detachment using poly-HEMA-coated plates (Cytone 1). After 24, 48, and 72 h, cells were collected, stained with trypan blue (Hyclone, Logan, UT, USA), and total cells and total dead cells were counted using a hemocytometer. This experiment was repeated three times for ECM-attached cells and ECM-detached cells.

2.6. Statistical Analysis

Statistical significance was determined using a two-tailed t-test for soft agar, cell proliferation, and cell viability assays where $p < 0.05$ was considered statistically significant. Error bars represent standard error of the mean (SEM).

3. RESULTS

3.1. SESN2 Deficiency Decreases Survival of Anchorage-Independent SKOV3 Cells

The soft agar assay tests the ability of cancer cells to form colonies in anchorage-independent environments similar to what is encountered during metastasis. To determine if SESN2 deficiency affected anchorage-independent growth of SKOV3 cells, SKOV3 cells were engineered to be deficient in SESN2 using shRNA techniques, and SESN2 deficiency was confirmed by western blot (**Figure 1A**). The soft agar assay was then used to determine if SESN2 deficiency affected anchorage-independent colony growth when compared to SKOV3 parental cells. Results showed that SESN2-deficient SKOV3 cells had significantly less colony formation than parental cells (**Figure 1B** and **1C**).

3.2. SESN2 Deficiency Compromises Cell Viability in ECM-Detached SKOV3 Cells

In order to determine if lack of colony formation in soft agar in SESN2-deficient cells was due to decreases in proliferation or changes in cell viability, SKOV3 parental cells and SKOV3 SESN2-deficient cells were plated on poly-HEMA-coated plates. Poly-HEMA plates do not allow for cells to deposit matrix; therefore, cells are grown in ECM detachment. When plated in detachment, SKOV3 SESN2-deficient cells displayed decreased cell viability at the 48- and 72-h periods when compared to the

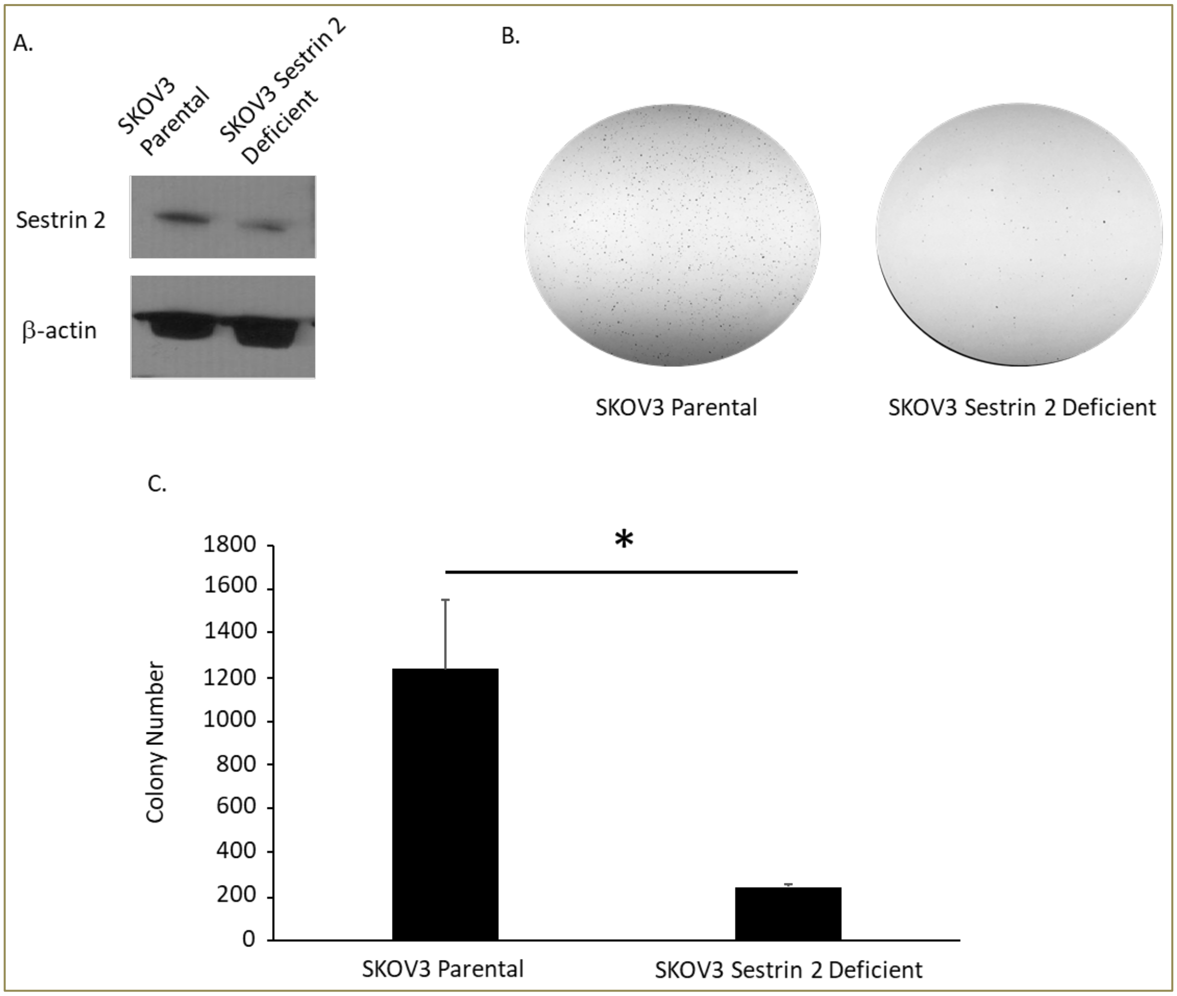

FIGURE 1. SESN2 deficiency in SKOV3 cells severely abrogates growth in soft agar. SESN2 deficiency was achieved in SKOV3 cells utilizing shRNA techniques and successful elimination was confirmed through western blotting (A). SKOV3 parental and SKOV3 SESN2-deficient cells were plated in triplicate in soft agar, fed every two days with 1 ml of media, and grown for 21 days. After 21 days, colonies were stained with iodonitrotetrazolium chloride (INT) and colonies were quantified using ImageJ software. Representative images from one individual experiment are in (B) and quantitation of colony number is shown in (C). Error bars represent SEM. *, $p < 0.05$.

SKOV3 parental cells (**Figure 2A**). However, there was no change in proliferation between ECM-detached SKOV3 parental cells and ECM-detached SKOV3 SESN2-deficient cells (**Figure 2B**). These data suggest that abrogated anchorage-independent growth visualized in SESN2-deficient SKOV3 cells is due to compromised cell viability rather than cell proliferation.

3.3. Attached SESN2-Deficient SKOV3 Cells Sustained Cell Viability and Proliferation

Noting that SESN2 deficiency abrogated cell viability in ECM-detached SKOV3 cells, we were interested to see if SESN2 deficiency affected cell viability and/or cell proliferation in ECM-attached SKOV3 cells. SKOV3 parental cells and SKOV3

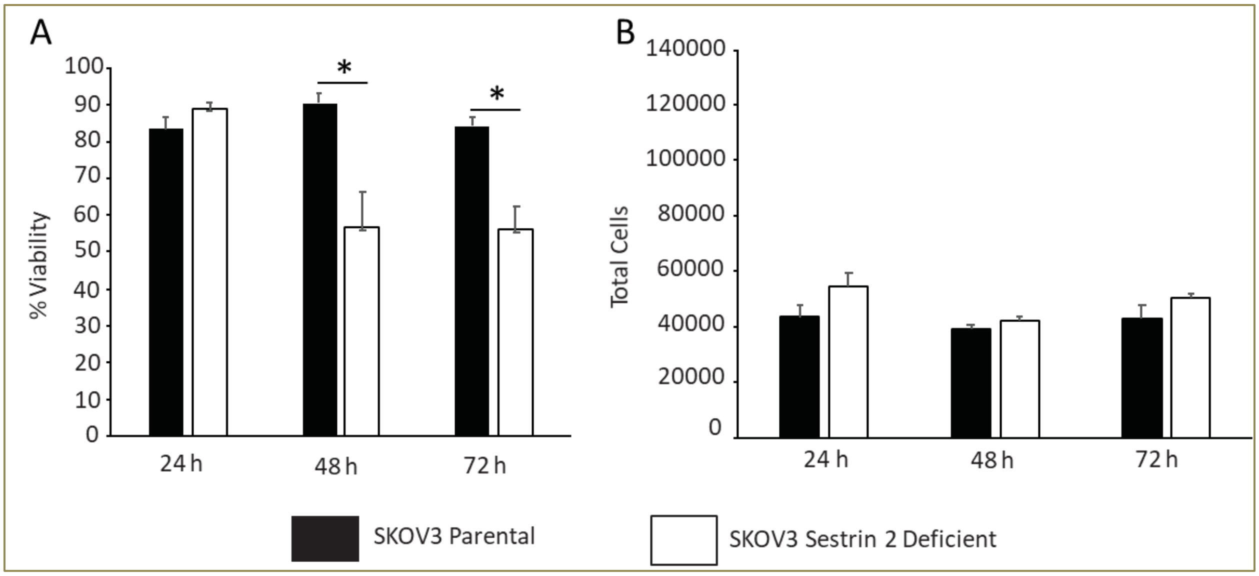

FIGURE 2. Elimination of SESN2 compromises cell viability in ECM-detached SKOV3 cells. SKOV3 parental and SKOV3 SESN2-deficient cells were plated in triplicate on non-adherent poly-HEMA-coated plates, and cells were analyzed at the 24, 48, and 72-h time points. This experiment was completed three times. Percent cell viability calculated at 24, 48, and 72-h time points is represented graphically in (A), where vertical bars represent the mean of all three experiments. Total proliferation at all three time points is shown in (B), and vertical bars represent the mean of all three experiments. Error bars represent the SEM. *, $p < 0.05$.

SESN2-deficient cells were plated on adherent 6-well plates, and counted at 24, 48, and 72-h time points. Interestingly, SESN2 deficiency had no effect on cell viability (**Figure 3A**) or cell proliferation (**Figure 3B**) in ECM-attached SKOV3 cells. All together, these results indicate that SESN2 deficiency has little impact on ECM-attached SKOV3 cells, suggesting that SESN2 deficiency plays little role in proliferation and cell viability at the primary tumor and a larger role during metastasis.

4. DISCUSSION

Here, our studies work to unravel the importance of SESN2 in the viability and proliferation in anchorage-independent SKOV3 epithelial ovarian cancer cells. We report that SESN2 plays a critical role in the colony formation of SKOV3 cells in soft agar. Specifically, SESN2 plays a unique and important role in maintaining cell viability in ECM-detached SKOV3 cells. Interestingly, SESN2 deficiency in ECM-attached cells displays no significant change in cell viability. Furthermore, SESN2 appears to play no role in maintaining proliferation in ECM-detached or ECM-attached SKOV3 cells (**Figure 4**).

The identification of SESN2 as key protein important in the viability of ECM-detached ovarian cancer cells adds to a growing body of literature that ROS-eliminating mechanisms are critical in metastatic cancers. Most recently, the antioxidant enzyme, catalase, was found to play a critical role in the viability of ECM-detached breast cancer cells and ECM-detached ovarian cancer cells [3, 11]. Furthermore, superoxide dismutase 2 (SOD2) has been implicated in breast cancer and nasopharyngeal anchorage-independent growth and metastasis [3, 12]. Here, we report that SESN2 plays a critical role in the viability of anchorage-independent EOC cells. Thus, novel therapeutic strategies aimed at SESN2 may be an effective strategy to eliminate ECM-detached metastatic EOC cells.

Our study is the first (to our knowledge) to study SESN2 in EOC and directly link SESN2 and the sur-

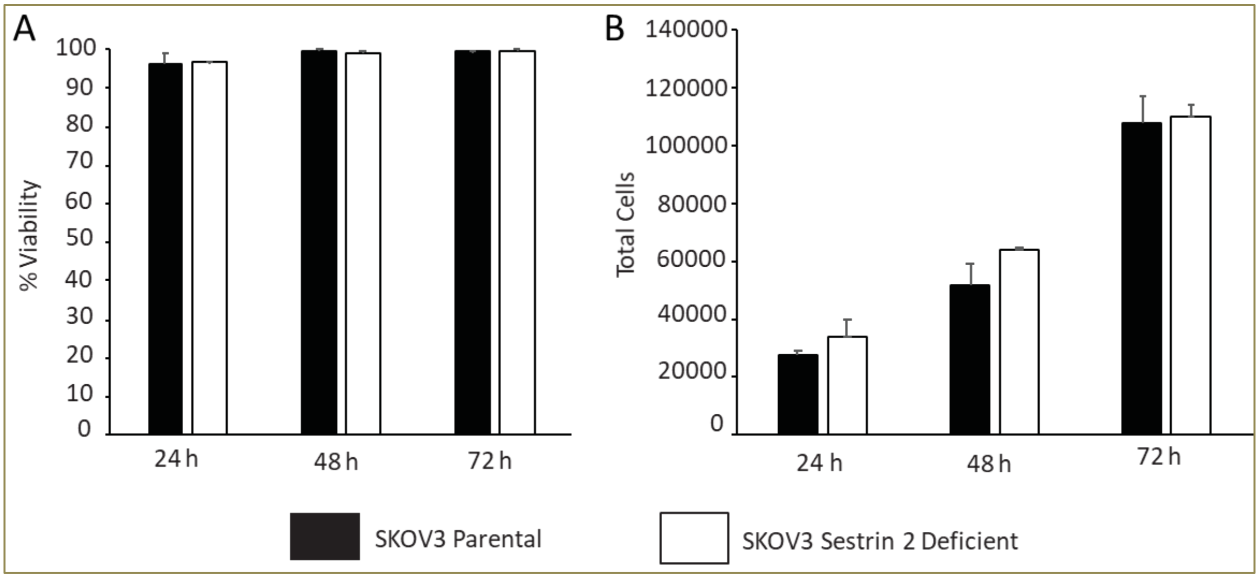

FIGURE 3. Elimination of SESN2 does not impact proliferation or cell viability of ECM-attached SKOV3 cells. SKOV3 parental and SKOV3 SESN2-deficient cells were plated on adherent plates in triplicate and counted after 24, 48, and 72 h. Percent viability is shown in (A) and total proliferation is represented graphically in (B). In both graphs, vertical bars represent the mean of three separate experiments and the error bars indicate SEM. No statistical significance was seen between ECM-attached SKOV3 parental cells and ECM-attached SKOV3 SESN2-deficient cells at any time point for cell viability or cell proliferation.

vival of ECM-detached EOC cells. The vast majority of studies have been aimed at understanding the role of SESN2 in basic physiological processes [13–18]; however, only recently has SESN2 been studied in cancer. As of late, it has been reported that decreased SESN2 expression has been connected to poor outcome in colorectal cancer [9], and induction of SESN2 through a variety of mechanisms has been connected with successful elimination of cancer cells [10, 19–22]. Interestingly, our study finds that elimination of SESN2 compromises viability of ECM-detached metastatic EOC cells. Thus, our study represents a novel paradigm whereby SESN2 expression actually promotes survival of ECM-detached metastatic EOC cells. In hepatocellular carcinoma, SESN2 expression was increased compared to the benign tissue, and this increased SESN2 expression was connected with resistance to the first-line therapy sorafenib [23]. Collectively, these studies reveal that the role of SESN2 is unique in many different contexts, and future studies aimed at elucidating the role of SESN2 in different contexts are warranted to better treat this disease.

In aggregate, it is plausible that SESN2 elimination could be a viable therapeutic tactic for specifically treating metastatic EOC. Given the low 5-year survival rate associated with late-stage cancer, it is crucial to work to develop better treatments for patients diagnosed with this disease [1]. Furthermore, it is of critical importance to study SESN2 expression levels in EOC tissues to determine if SESN2 could serve as an improved diagnostic biomarker.

5. CONCLUSION

Here, we report SESN2 deficiency compromises growth of ECM-detached SKOV3 EOC cells in the soft agar assay. Furthermore, this decreased colony formation is the result of decreased viability in SESN2-deficient SKOV3 cells. Interestingly, elimination of SESN2 in ECM-attached cells showed no

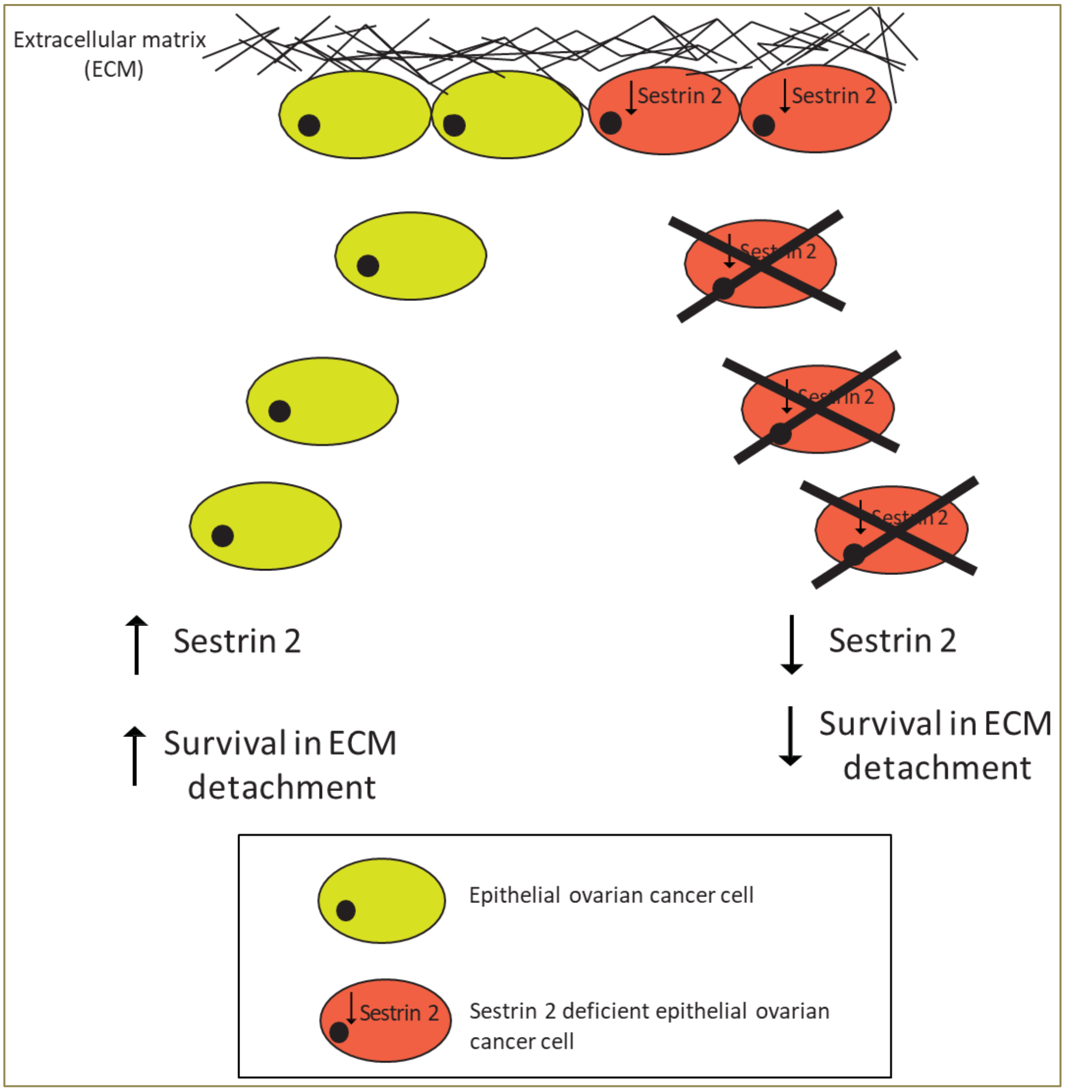

FIGURE 4. A proposed model for the role of SESN2 in SKOV3 epithelial ovarian cancer cells. Here, we report that while SESN2 deficiency has no impact on ECM-attached epithelial ovarian cancer cells at the primary tumor, SESN2 deficiency in ECM-detached epithelial ovarian cancer cells results in severely abrogated cell viability. Given that survival in ECM detachment is necessary for metastasis, these data indicate that SESN2 expression is important for maintaining cell viability and is important for successful metastasis of epithelial ovarian cancer cells. Therefore, SESN2 expression is critical for survival and metastasis of epithelial ovarian cancer cells.

changes in proliferation or viability, suggesting that SESN2 plays a unique and critical role in the growth of ECM-detached SKOV3 cells. Therefore, SESN2 is a plausible target for treating metastatic EOC and requires further investigation.

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