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The interaction of Hemin and Sestrin2 modulates oxidative stress and colon tumor growth

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Abstract

Several large epidemiological and animal studies demonstrate a direct correlation between dietary heme iron intake and/or systemic iron levels and cancer risk in several cancers including colorectal cancer (CRC). However, the precise mechanisms for how heme iron contributes to CRC and how cancer cells respond to heme iron-induced stress are still unclear. Previously we have shown that one of the stress-inducible proteins, Sestrin2 (SESN2), is a novel tumor suppressor in colon by limiting endoplasmic reticulum stress and mammalian target of rapamycin complex 1 (mTORC1) signaling and tumor growth. But the relationship between heme iron and SESN2, especially in the context of colon carcinogenesis, was not investigated previously. Here, we found that hemin dose-dependently increased SESN2 expression in an oxidative stress and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2, NRF2)-dependent manner. Since SESN2 overexpression reduced hemin-induced oxidative stress, SESN2 could be an important target of NRF2 exerting antioxidant function. Indeed, expression of several oxidative stress responsive proteins such as NRF2 and its target genes was reduced by SESN2. Although we formerly reported that SESN2 expression was reduced after p53 mutation in colon tumors, mouse colon tumors, which have intact p53 and NRF2, induced SESN2 expression in response to iron stimulus. Although SESN2 overexpression decreased murine colon tumor cell growth both in vitro and in vivo, it rendered colon cancer cells more resistant to hemin-induced apoptosis and therefore promoted tumor growth during hemin treatment. Taken together, although SESN2 generally suppresses tumorigenesis, it can produce tumor-promoting role in iron-rich environment by suppressing oxidative stress-associated cancer cell death.

Keywords

Sestrin2; Hemin; Oxidative stress; Colorectal cancer; Nuclear factor (erythroid-derived 2)-like 2

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Conflict of interest statement

The authors have no financial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2019.04.025.

1. Introduction

Colorectal cancer (CRC) is the second most prevalent cancer and the second leading cause of cancer-related deaths when men and women are combined in the United States (Bray et al., 2018). Understanding the mechanisms of growth and progression of CRC is essential to improve treatment. Consumption of red meat, which is enriched with heme, is associated with significant risk of developing CRC (Sandhu et al., 2001; Larsson and Wolk, 2006). Heme may explain the relationship between colon cancer and red meat (eg. beef, pork, lamb) intake since no link between white meat (e.g. fish, chicken) intake and CRC has been found in either men or women (Sesink et al., 1999; Willett et al., 1990; Giovannucci et al., 1994). Furthermore, heme iron is sufficient to promote colon tumorigenesis (Bastide et al., 2015). Dietary heme is predominantly from hemoglobin and myoglobin in meats and can be directly absorbed in the small intestine (Oates and West, 2006; West and Oates, 2008). Heme carrier protein-1 (HCP1) was proposed to be responsible for the intestinal heme absorption, though later studies indicate that it might not be a physiologically relevant heme transporter (West and Oates, 2008). At the cellular level, endocytosis of hemoproteins linked with heme-responsive gene-1 (HRG1) imports extracellular free heme into colonic epithelia (Rajagopal et al., 2008; Gulec et al., 2014; Shayeghi et al., 2005; White et al., 2013).

Heme (ferroprotoporphyrin, Fe^{2+}) is a tetrapyrrole ring containing an atom of ferrous iron in its center, whereas hemin (ferriprotoporphyrin, ferric heme, Fe^{3+}) contains a ferric iron, which can be further oxidized yielding ferryl species (Kagan et al., 2001; Itoh et al., 2001). Administration of hemin to macrophages leads to intracellular accumulation of ferrous heme (Mullebner et al., 2017). Heme-containing enzymes are critical for many biological processes such as mitochondrial respiration (e.g. cytochrome C), oxygen transport and storage (e.g. hemoglobin and myoglobin), detoxification (e.g. cytochrome P450), antioxidant defenses (e.g. catalase, peroxidase), and signal transduction processes (e.g. nitric oxide synthase) (Nagababu and Rifkind, 2004; Ryter and Tyrrell, 2000; Shimizu, 2012). Hemin is genotoxic and cytotoxic to human colon cells (Glei et al., 2006; Gemelli et al., 2014).

Our previous study has also shown that the major intestinal iron uptake transporter divalent metal transporter (DMT) 1 is highly increased in colon tumors by hypoxia inducible factor-2α (Xue et al., 2012). Moreover, pharmacological inhibition or genetic disruption of colonic DMT1 decreases colon tumorigenesis via iron/cyclin-dependent kinase 1/Janus kinas 1/signal transducer and activator of transcription 3 signaling pathway (Xue et al., 2016). Mitochondrial iron accumulation, oxidative stress and colon tumorigenesis are increased by intestine-specific overexpression of six-transmembrane epithelial antigen of prostate 4 (STEAP4), a ferrireductase which is highly increased in CRC. In contrast, STEAP4-enhanced colon tumorigenesis is reduced when deferiprone was used to chelate mitochondrial iron in mice (Xue et al., 2017). These studies suggest that iron homeostasis is critical for colon carcinogenesis. However, the response of cancer cells to heme iron stress is not fully understood.

A recent study showed that heme and iron induced a cellular protein aggregation in response to oxidative stress in macrophage (Vasconcellos et al., 2016). Cellular stress response, which

is a defensive reaction of cells to environmental damage, plays a critical role in redox sensing and regulation, DNA damage repair and cell cycle control (Kultz, 2005). Sestrins (SESNs) are a family of proteins that are highly conserved and are upregulated in cells under oxidative stress and metabolic restriction (Seo et al., 2015; Hwang et al., 2018; Byun et al., 2017). SESN proteins consist of three distinct family members, SESN1, SESN2, and SESN3 (Pasha et al., 2017). SESN1 (also named as p53 activated gene 26, PA26) was first identified as a novel target gene of p53 induced by genotoxic stress and has the property of the growth arrest DNA damage (GADD) gene family (Velasco-Miguel et al., 1999). SESN2 (also named as hypoxia induced gene #95, Hi95) was discovered to be induced by prolonged hypoxia, oxidative stress or DNA damage (Budanov et al., 2002). In contrast, SESN3 is regulated by RAS GTPase-activating protein and forkhead box protein O but not p53 to modulate intracellular reactive oxygen species (ROS) levels (Kopnin et al., 2007; Nogueira et al., 2008). During oxidative stress, SESNs suppress oxidative stress through various mechanisms (Ho et al., 2016), including direct oxidoreductase activity (Kim et al., 2015), autophagic degradation of kelch-like ECH-associated protein1 (KEAP1) and activation of NRF2 (Bae et al., 2013; Rhee and Bae, 2015; Ro et al., 2014) and dysfunctional mitochondria (Lee et al., 2010). Previously, we and others have shown that loss of SESN2 promotes colon tumor growth through increased endoplasmic reticulum stress and p53-mediated control over mTORC1 signaling (Ro et al., 2016; Budanov and Karin, 2008; Peng et al., 2014). Here, we tested the hypothesize that hemin can activate SESN2 by increasing ROS and NRF2 in CRC. Also, the interaction of hemin and SESN2 on colon tumor development was assessed in vitro and in vivo.

2. Materials and methods

2.1. Cell culture

Human HCT116, RKO, and murine MC38 CRC cells were maintained at 37 °C in 5% CO_2 and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (VWR, Radnor, PA).

2.2. Animals

All mice were maintained in a standard cage in light and temperature-controlled room and were allowed standard chow and water excepted as indicated. Animal studies were performed in accordance of the Institute of Laboratory Animal Resources guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center (Protocol# HSC-18–200699) and followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). A colon-specific tumor model driven by the tamoxifen-inducible caudal type homeobox 2 (CDX2) ^{ERT2}-Cre promoter and monoallelic flox of the frequently mutated adenomatous polyposis coli (*APC*) gene in CRC patients ($CDX2^{ERT2}Apc^{F/+}$), were utilized and treated as previously described (Xue et al., 2016). To be noted, the p53 gene is wildtype in this model. Briefly, $CDX2^{ERT2}Apc^{F/+}$ mice ($n = 18$) were treated with 100 mg/kg tamoxifen for 3 consecutive days, and then one day later were given AIN93G diet containing 1000 mg/kg of iron (1000Fe, high-iron diet) or iron sufficient AIN93G diet containing 35 mg/kg of iron (35Fe, control-iron diet, Dyets, Bethlehem, PA).

Two days after the initiation of iron diet treatment, these mice were treated with 1.5% dextran sulfate sodium (DSS) for 7 days (inflammatory phase). Thereafter mice were placed on regular drinking water for 14 days (recovery phase), and one more inflammatory and recovery cycles were performed to establish the CRC model. CDX2 $Apc^{F/+}$ mice ($n = 14$), in which colon tumors are developed spontaneously with aging, were sacrificed at 3 months old. For subcutaneous xenograft study, to test the role of SESN2 under immuneintact condition, parental MC38 and MC38 syngeneic cells with SESN2 overexpression were treated with or without 100 μM hemin (Sigma, St Louis, MO) overnight, trypsinized and resuspended in sterile 1× phosphate-buffered saline (PBS). Cells were counted and diluted with 1× PBS to a concentration 1×10^7cells/mL, and then 100 μl containing 1×10^6 total cells were injected into the flanks of C57BL/6 mice ($n = 13$). Two weeks later, mice were sacrificed and tumors were collected. Both male and female animals were used in this study and no influence of sex on the results of the study was observed.

2.3. Western blot analysis

Cells or tumor tissues were lysed with radioimmunoprecipitation assay buffer and incubated on ice for 30 min. After incubation, cell extracts were centrifuged for 10 min at 4 °C. The supernatant was collected for Bradford assay to quantify protein concentration. Equal amounts (30 μg-50 μg) of protein were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), along with molecular weight marker. The gels were run for 1 h 30 min at 120 V. The proteins inside the gels were transferred onto nitrocellulose membrane for 1 h at 18 V using semi-dry transfer method. The membranes were blocked with 3% milk for 1 h. Primary antibodies against SESN2, Flag, ferritin heavy chain (FTH1), nuclear factor-like 2 (NRF2), cleaved caspase 3 (cCasp3), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA), green fluorescence protein (GFP), NAD(P)H Quinone Dehydrogenase 1 (NQO1), heme oxygenase-1 (HO-1), proliferating cell nuclear antigen (PCNA) and cyclin D1 (CCND1) (Santa Cruz Biotechnology, Dallas, TX) were used. Secondary antibodies against Rabbit (Cell signaling Technology, Danvers, MA) and Mouse IgG (Cell signaling Technology, Danvers, MA) were used.

2.4. 2′,7′-Dichlorofluorescein diacetate (DCFDA) staining

Cells (2×10^5 cells/well) were plated into 24 well plates. After 24 h, drugs were added at indicated concentrations. After 24 h, the cells were washed one time with DMEM. Pre-warmed DMEM containing 10uM DCFDA (Cayman Chemical Company, Ann Arbor, MI) was added and the cells were incubated at 37 °C for 30 min. The cells were washed once with DMEM and twice with 1× PBS. Representative images were taken using the GFP channel of an Invitrogen™ EVOS™ FL Auto Imaging System (Thermo Fisher Scientific). Fluorescence intensity was quantified using a SpectraMax M2 Microplate Reader (Molecular Devices, Radnor, PA) at excitation 485 nm and emission 530 nm. The intensity was normalized with protein concentration.

2.5. Transfection

For transient overexpression, pLU-CMV-Flag-hSESN2 WT or empty plasmids were transfected into HCT116 and RKO using polyethylenimine (PEI). Experiments were carried

out 24 hours post-transfection. For stable overexpression, MC38 cells were transfected with pLC242-GFP-SESN2 plasmid (#100519, Addgene) and selected with 1 μg/ml blasticidin.

2.6. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted using IBI Isolate DNA/RNA Reagent Kit (IB47602, IBI Scientific, Dubuque, IA). qPCR was performed using a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). The following pre-designed primers were used: mouse *Sesn2* forward TCC GAGTGCCATTCCGAGAT, mouse *Sesn2* reverse TCCGGGTGTAGACCC ATCAC, 18S forward GTAACCCGTTGAACCCCATT and 18S reverse CCATCCAATCGGTAGTAGCG.

2.7. Thiazolyl blue tetrazolium bromide (MTT) assay

Cells were plated at a concentration of 5×10^4 cells/mL in a 24 well plates. 125 μL 5 mg/mL MTT (Sigma, MO) was added to each plate and incubated for 30 min. Dimethyl sulfoxide (DMSO) was added and absorbance was measured at 570 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, Molecular Devices, Radnor, PA).

2.8. Statistical analysis

Data were expressed as mean ± SD. p values were calculated by independent t-test, one-way and two-way analysis of variance (ANOVA). $p < .05$ was considered significant.

3. Results

3.1. SESN2 is dose-dependently increased by hemin in CRC cells

To test the effect of hemin on SESN2 expression, HCT116 and RKO CRC cells were treated with different physiologically relevant concentrations of hemin (0–100 μM) (Goldstein et al., 2003) (Fig. 1). The initial reason we chose these two cell lines is that both of these cell lines express wild-type p53 and therefore the basal level of SESN2 expression is detectable (Ro et al., 2016). The doses chosen for hemin are based on a recent report that male 4-week-old F344 rats fed with a 1% hemoglobin diet for 100 days resulted in 200–300 μM heme in rat fecal water (Bastide et al., 2015). Hemin can be catabolized into equimolar amounts of iron, carbon monoxide and biliverdin by the enzyme HO-1 (Abraham and Kappas, 2008; Gozzelino et al., 2010). Carbon monoxide and biliverdin are cytoprotective, but iron can be toxic due to generation of ROS through Fenton reaction. Thus, the ferroxidase and iron storage protein FTH1 is induced to bind and oxidize iron to avoid the detrimental effect. As expected, FTH1 was increased by hemin in both HCT116 and RKO cell lines (Fig. 1A and C). Interestingly, SESN2 was also dose-dependently increased by hemin (Fig. 1A and B). SESN2 is induced in ulcerative colitis but is downregulated in CRC in a p53-dependent manner (Ro et al., 2016). However, hemin can lead to the nuclear export and cytosolic degradation of p53 (Shen et al., 2014). Since hemin can suppress p53 activity, SESN2 induction in HCT116 and RKO cell lines should be through a mechanism that is independent of p53.

3.2. Hemin-induced SESN2 is dependent on oxidative stress and NRF2 in CRC cells

We searched for a possible mechanism of how SESN2 is induced upon hemin challenge. Previous reports showed that SESN2 was highly upregulated by H_2O_2 in different cell lines (Liu et al., 2017; Ishihara et al., 2013). Consistently, we found here that SESN2 was dose-dependently upregulated by H_2O_2 in both HCT116 and RKO CRC cells (Fig. S1A and S1B). Heme is known to induce ROS through the activation of nicotinamide adenine dinucleotide phosphate oxidase or via the mitochondria (Barcellosde-Souza et al., 2013; Hasan and Schafer, 2008; Dutra et al., 2014). To assess whether hemin can indeed increase ROS generation, DCFDA staining was performed. DCFDA is a cell-permeable fluorogenic probe to quantify intracellular ROS (Owusu-Ansah et al., 2008). It is rapidly oxidized by ROS species such as nitric oxide and H_2O_2 in cells to form fluorescent 2′,7′-dichlorofluorescein (Gabriel et al., 1997). We found that hemin dose-dependently increased green fluorescence indicating increased oxidative stress levels in both cell lines (Fig. S2A and S2B). To further determine whether hemin-induced SESN2 is dependent on oxidative stress, N-Acetyl Cysteine (NAC) was utilized. NAC is a widely used thiol-containing antioxidant and generates reduced glutathione (Aldini et al., 2018). It interacts with OH of H_2O_2, thus decreasing ROS levels. Previously, NAC inhibited hemin-induced ROS surge and cellular stress response in vascular smooth muscle cells (Hasan and Schafer, 2008). To confirm that hemin-induced ROS levels were decreased by NAC, DCFDA staining was performed. NAC and hemin co-treatment generated less green fluorescence intensity compared to hemin-only treatment (Fig. 2A and B). At the same time, we found that NAC significantly reduced hemin-increased SESN2 expression in both HCT116 and RKO cells (Fig. 2C and D).

It is well established that the increase of ROS production is accompanied with the induction of a large number of cellular antioxidant genes via transcriptional activation of the antioxidant response elements (ARE) in their promoters (Nguyen et al., 2009). NRF2 is a master transcriptional regulator that binds with ARE and activates antioxidant gene expression (Pall and Levine, 2015). Previously, it has been shown that NRF2-ARE pathway induces SESN2 (Shin et al., 2012). Here we found that ML-385, a NRF2 specific inhibitor that binds to the NRF2 DNA binding domain and interferes its transcriptional activity (Singh et al., 2016), abolished hemin-induced SESN2 expression (Fig. 3A and B). These results indicate that hemin induces SESN2 through inducing oxidative stress and subsequent activation of NRF2 transcription factor.

3.3. SESN2 overexpression reduces hemin-induced oxidative stress and the expression of oxidative stress responsive proteins

To understand the role of SESN2 induction during hemin-induced oxidative stress, SESN2 was overexpressed in both HCT116 and RKO cells. Given that SESN2 has antioxidant properties (Byun et al., 2017) and overexpression of SESN2 inhibited glucose deprivation-induced ROS production (Seo et al., 2015), DCFDA staining was also applied. Consistently, hemin increased the DCFDA fluorescence intensity in RKO cells, which was dramatically decreased by SESN2 overexpression in RKO cells (Fig. 4A and B). Thus, SESN2 can reduce hemin-induced oxidative stress. As expected, the expression of SESN2 was dramatically increased by the SESN2 plasmid (Fig. 4C). Consistent with a previous report (Vasconcellos et al., 2016), we found that NRF2 was increased by hemin. However, hemin-induced NRF2

expression was repressed by SESN2 overexpression (Fig. 4C and Fig. S3A). Similarly, the expression of two NRF2 targets and antioxidant proteins, NQO1 and HO-1, were induced by hemin and decreased by SESN2 overexpression (Fig. 4C, Fig. S3B and S3C). This is consistent with known function of SESN2 in downregulating oxidative stress. Together, SESN2 can reduce hemin-induced oxidative stress response.

3.4. Sesn2 expression is increased in mouse colon tumors

To test the expression of SESN2 in vivo, normal and tumor colon tissues were collected from the colitis-associated and iron-driven CDX2^{ERT2}*Apc*$^{F/+}$ CRC mouse model as described previously (Xue et al., 2016). Interestingly, in the control-iron diet group (35 Fe), the mRNA expression of Sesn2 was significantly increased in the tumor colon tissues compared to normal colon tissues, which was further enhanced by high-iron diet (1000 Fe) treatment (Fig. 5A). To tease out the effect of DSS-induced inflammation on the expression of Sesn2, the sporadic CRC model of CDX2 *Apc*$^{F/+}$ mice were also tested. Similarly, the mRNA expression of Sesn2 was increased in the tumor colon tissues compared to normal colon tissues (Fig. 5B). Together, the mRNA expression of Sesn2 is increased in mouse colon tumors and increased by heme iron.

3.5. SESN2 overexpression and hemin modulates colon tumor cell growth

In addition to its pro-oxidative and cytotoxic effects, heme also activates innate immune responses and inflammation (Dutra et al., 2014). Additionally, SESN2 has been shown to suppress proinflammatory signaling in macrophages (Yang et al., 2015). Thus, we tested the roles of hemin and SESN2 in colon tumor growth using the syngeneic murine MC38 CRC cells, which can grow into tumors under an immune-sufficient environment in vivo. To test the direct role of SESN2 on colon tumor cell growth, MTT assay was first conducted in MC38 cells with SESN2 overexpression. SESN2 overexpression in MC38 cells was confirmed by Western blot analysis (Fig. 6A). SESN2 overexpression significantly reduced the cell survival rate compared to the empty vector control group (Fig. 6B). Next, MC38 cells were injected into C57BL/6 mice to test the effect of SESN2 overexpression in vivo. After subcutaneously injection, MC38 cells developed into large tumor (> 1 cm) within 2 weeks (Fig. 6C). The developed tumor weight was significantly lower in the SESN2 overexpression group compared to the control group (Fig. 6D).

To test the role of hemin on colon tumor growth, we treated MC38 cells with hemin overnight before the subcutaneous injection (Fig. 7). Interestingly, hemin treatment significantly increased the resulting tumor size of SESN2 overexpressing MC38 cells (Fig. 7A). Hemin treatment didn't affect the tumor weight in the control MC38 cells, whereas SESN2 overexpression consistently reduced tumor weight (Fig. 7B). Western blot analysis showed that the expression of cCasp3, a marker of apoptosis, was not changed by hemin treatment in the tumor formed by control MC38 cells or by SESN2 overexpression. However, hemin treatment of SESN2 overexpression cells significantly reduced the expression of cCasp3 in the formed tumor compared to non-treated control cells (Fig. 7C and D). In contrast, the cell proliferation marker PCNA and the cell cycle related gene CCND1 were not changed either by hemin or SESN2 overexpression in the tumor formed by

MC38 cells (Fig. 7C, E and F). Together, these data demonstrate that SESN2 has a dual role on tumor cell growth.

4. Discussion

Our data herein shows that hemin can activate SESN2 via inducing ROS and NRF2, whereas oxidative stress can be reduced by SESN2 overexpression. Also, we have demonstrated that SESN2 was highly upregulated in colon tumors from both colitis-associated CRC model CDX2^{ERT2}*Apc*$^{F/+}$ mice and sporadic CRC model CDX2 *Apc*$^{F/+}$ mice. Furthermore, we found SESN2 overexpression can decrease tumor growth under normal iron conditions. However, surprisingly, SESN2 overexpression together with hemin treatment leads to an increase in tumor growth, indicating a complicated scenario under the high iron condition.

Previously, hemin has been reported to reduce the expression of p53 (Shen et al., 2014), which is known to regulate the expression of SESN2 (Ro et al., 2016). Interestingly, here we found that SESN2 was increased by hemin in colon epithelial cells. It has been reported that the induction of SESN2 by DNA damaging treatments occurs in a p53-dependent manner, whereas its induction in response to prolonged hypoxia or by oxidative stress is p53-independent (Budanov et al., 2002). Akt signaling mediated SESN2 activation is also independent of p53 (Ben-Sahra et al., 2013). Also, in contrast to reduced expression of SESN2 in human colon tumors (Ro et al., 2016), here we found an increased expression of SESN2 in mouse colon tumors. This may be due to the fact that there is increased hypoxia signaling (Xue et al., 2016) and a lack of p53 mutation in these animal models. It would be interesting to see whether chronic hemin treatment will eventually lead to decreased expression of p53 and SESN2.

A previous report showed that SESN2 activates NRF2 through p62 dependent autophagic degradation of KEAP1 triggered by the acute lipogenic stimulus in the liver (Bae et al., 2013). Under normal conditions, KEAP1-Cullin3-E3 Ubiquitin ligase complex mediates the poly-ubiquitination and proteasomal degradation of NRF2 (Ma, 2013). Here we found that SESN2 overexpression repressed the expression of NRF2 and its targets NQO1 and HO-1 (Loboda et al., 2016). These results indicate that SESN2 may modulate NRF2 expression through KEAP1 independent mechanism (Bryan et al., 2013).

The effect of SESN2 on cell viability is complicated. Previous reports have shown that SESN2 overexpression leads to inhibited cell growth and proliferation in many cultured cells, but it also protects against apoptosis caused by hypoxia or H_2O_2 in cancer cells and oxidized low-density lipoprotein in macrophages (Budanov et al., 2002, 2004; Hu et al., 2015). Recently it has been reported that SESN2 induction is essential for cancer cell survival upon glutamine depletion (Byun et al., 2017) or under glycolysis inhibition induced energetic stress (Ben-Sahra et al., 2013). In our study, we found that SESN2 reduced hemin-induced ROS in CRC cells and suppressed colon tumor growth in the absence of hemin, but the colon tumor growth was enhanced in the presence of both hemin and high levels of SESN2. To be noted, we acutely treated MC38 cells with hemin overnight before the subcutaneously xenograft. Aqueous solutions of hemin can be very unstable, with a half-life of a few hours (Cannon et al., 1995). However, addition of antioxidants can reduce radicals,

and led to slower degradation process and increased stabilization of hemin. This may partly explain why the increased tumor weight and reduced cCasp3 was only observed in the presence of both hemin and a high level of SESN2. It has been recently reported that dietary heme iron but not systemic heme promoted colon tumorigenesis (Constante et al., 2017), therefore we may need to put our mice on heme iron rich diet and then perform orthotropic transplantation of SESN2 overexpression MC38 cells into the cecum of mice. This may be more accurate in assessing the combinational effect of hemin and SESN2 on colon tumor growth under a more physiological condition.

A previous study has shown that hemin counteracts the cell death effects of imatinib via repressing NRF2 in leukemia cells (Bonovolias and Tsiftsoglou, 2009). NRF2 knockdown suppresses hypoxia activated angiogenesis and colon tumor growth in the HT29 and HCT116 xenograft mouse models (Kim et al., 2011). However, Nrf2 knockout mice crossed with *Apc*$^{min/+}$ mice or treated with azoxymethane and DSS decreases the antioxidative enzymes including NQO1, potentiates inflammation and enhances intestinal carcinogenesis (Cheung et al., 2014; Khor et al., 2008). Thus, it would be interesting to see the colon tumor growth rate after SESN2 depletion or NRF2 induction under a high hemin condition.

In summary, we have found that hemin induces SESN2 via ROS, which is a protective mechanism for suppressing oxidative stress and colon tumor growth, while high amount of SESN2 also protects hemin-induced cell death and leads to increased tumor growth (Fig. 8). Thus, similar to NRF2 (Tong et al., 2015; Gonzalez-Donquiles et al., 2017; Sebens et al., 2011), SESN2 may have dual roles in tumor suppression as well as tumor promotion depending on the context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

ARE	antioxidant response elements
cCasp3	cleaved caspase 3
CCND1	cyclin D1

CRC	colorectal cancer
DCFDA	2′,7′-Dichlorofluorescein diacetate
DMSO	dimethyl sulfoxide
DMT	divalent metal transporter
DSS	dextran sulfate sodium
EV	empty vector
FTH1	ferritin heavy chain
GADD	growth arrest DNA damage
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescence protein
HCP1	Heme carrier protein-1
HO-1	heme oxygenase-1
HRG1	heme-responsive gene-1
H_2O_2	hydrogen peroxide
KEAP1	kelch-like ECH-associated protein1
mTORC1	mammalian target of rapamycin complex 1
NAC	N-Acetyl Cysteine
NFE2L2 or NRF2	nuclear factor (erythroid-derived 2)-like 2
NQO1	NAD(P)H Quinone Dehydrogenase 1
PCNA	proliferating cell nuclear antigen
ROS	reactive oxygen species
SESN2	Sestrin2
STEAP4	six-transmembrane epithelial antigen of prostate 4.

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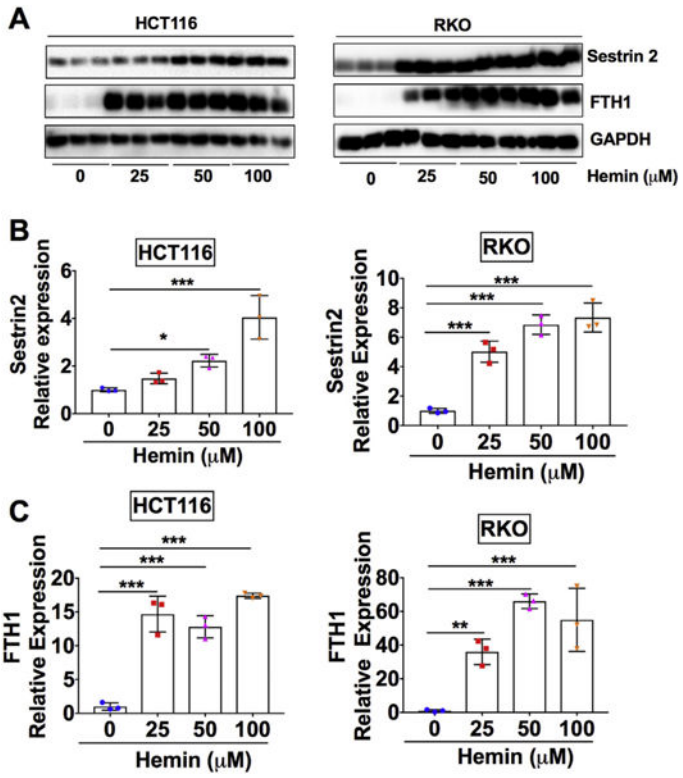

Fig. 1.

SESN2 is dose-dependently increased by hemin in CRC cells.

(**A**) Western blot analysis and (**B and C**) quantification in HCT116 and RKO CRC cells. Cells were treated with different doses of hemin as indicated for overnight. *$p < .05$, **$p < .01$ and ***$p < .001$.

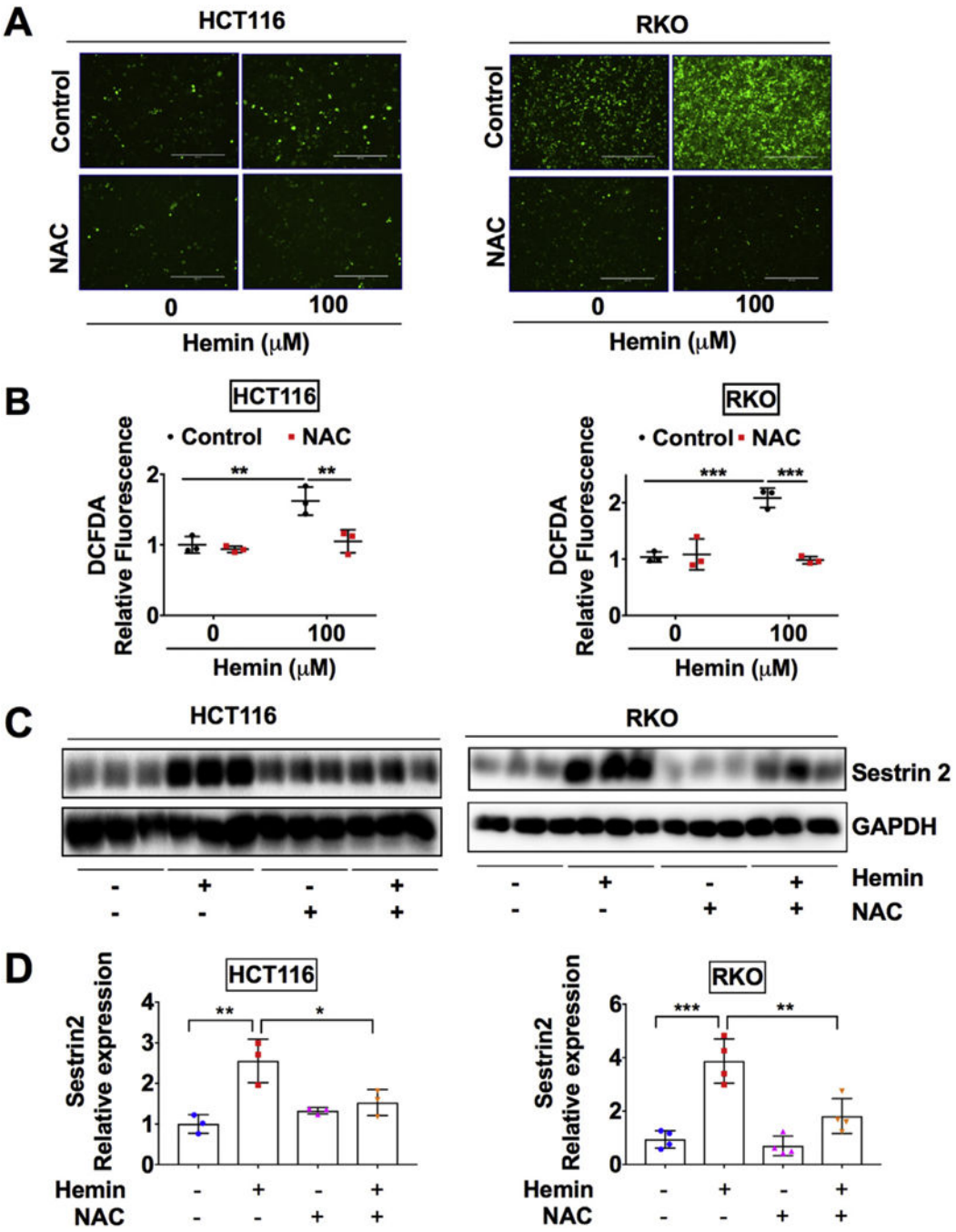

Fig. 2.

Hemin induced oxidative stress and SESN2 can be reduced by NAC in CRC cells.

(**A**) Representative green fluorescence images, (**B**) quantification of DCFDA staining, (**C**) Western blot analysis and (**D**) quantification in HCT116 and RKO CRC cells. Cells were treated with 100 μM hemin or 1 mM NAC as indicated for overnight. *$p < .05$, **$p < .01$ and ***$p < .001$.

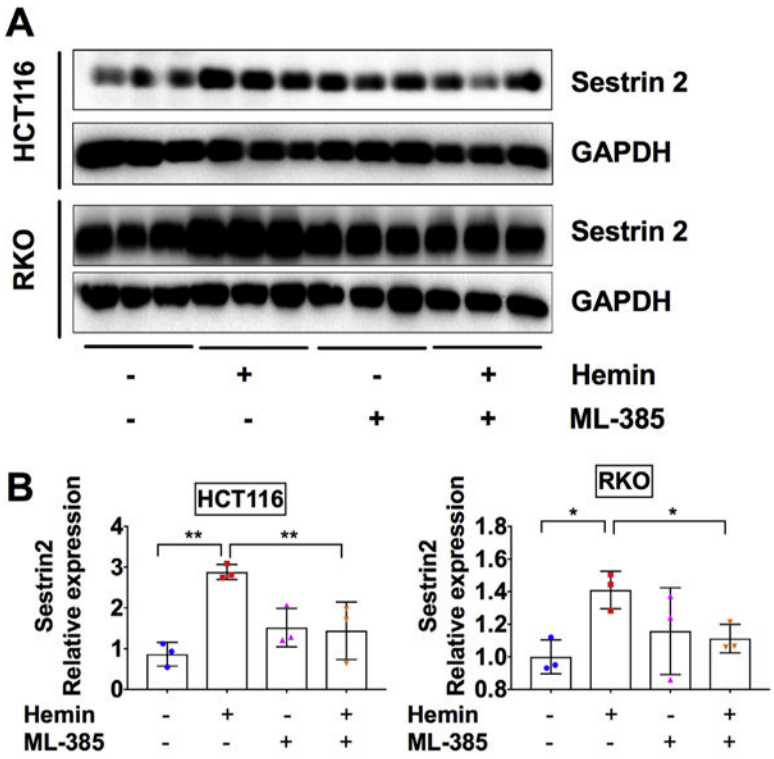

Fig. 3.

SESN2 induction by hemin is dependent on NRF2 activity in CRC cells.

(**A**) Western blot analysis and (**B**) quantification in HCT116 and RKO CRC cells. Cells were treated with 100 μM hemin or 5 μM NRF2 inhibitor ML385 as indicated for overnight. **p* < .05 and ***p* < .01.

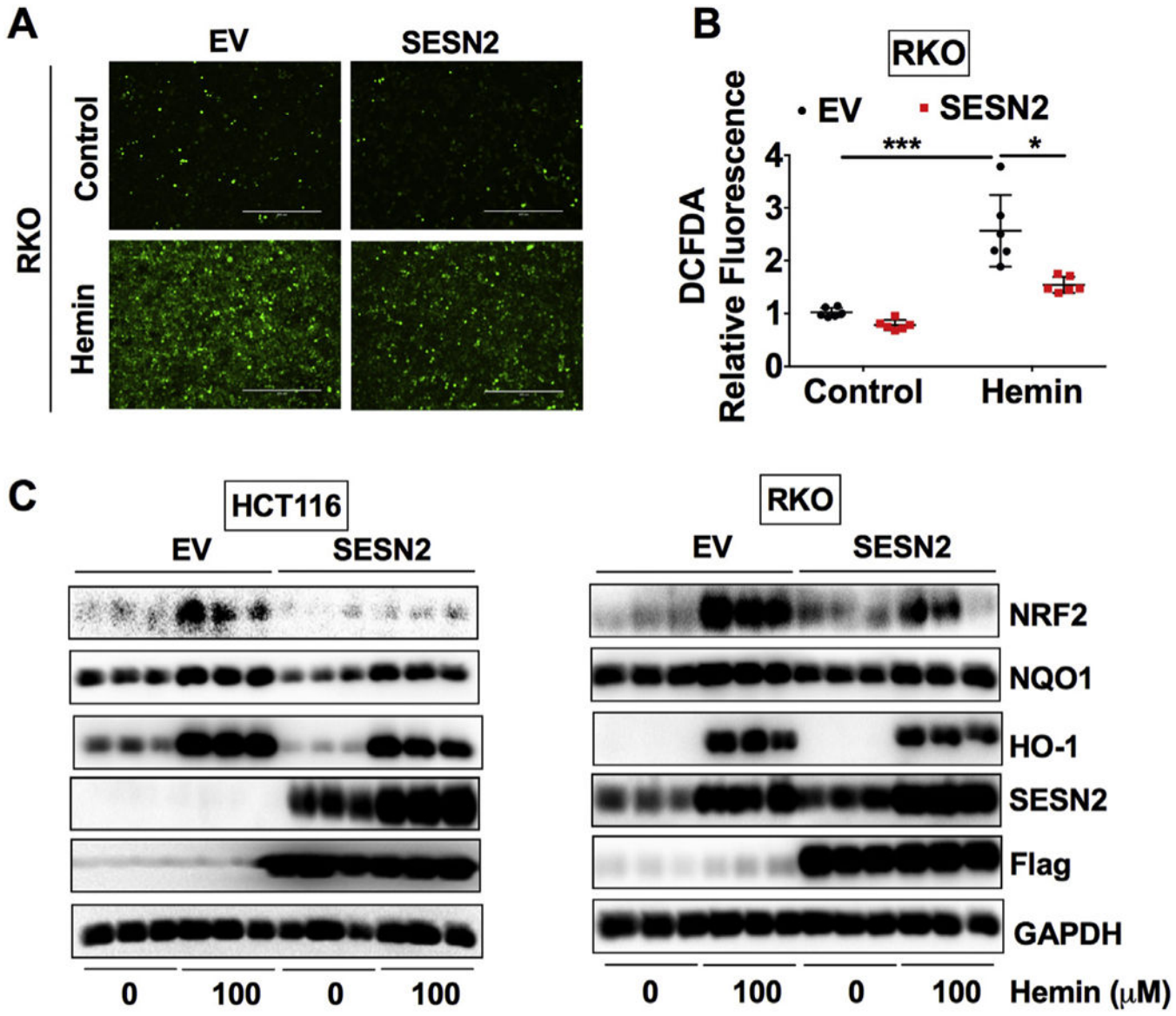

Fig. 4.

SESN 2 overexpression reduces the expression of hemin-enhanced oxidative stress responsive proteins in CRC cells.

(**A**) Representative green fluorescence images, (**B**) quantification of DCFDA staining and (**C**) Western blot analysis in HCT116 or RKO cells. Cells were transfected with human SESN2 plasmid or empty vector (EV) for 24 h and then these cells were treated with or without 100 μM hemin for overnight. $*p < .05$ and $***p < .001$.

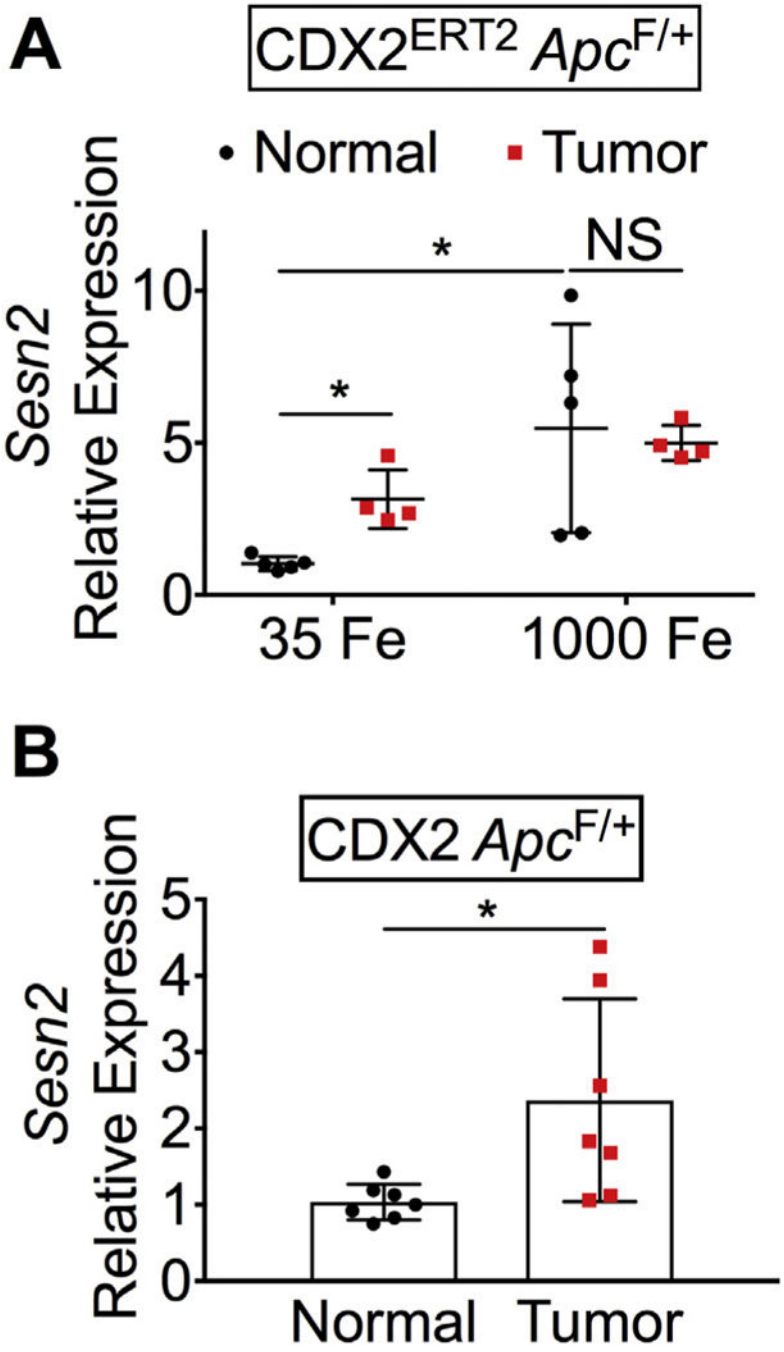

Fig. 5.

SESN2 expression is increased in mouse colon tumors.

qPCR analysis in (**A**) CDX2^{ERT2}*Apc*$^{F/+}$ mice and (**B**) CDX2 *Apc*$^{F/+}$ mice. *$p < .05$; NS, not significant.

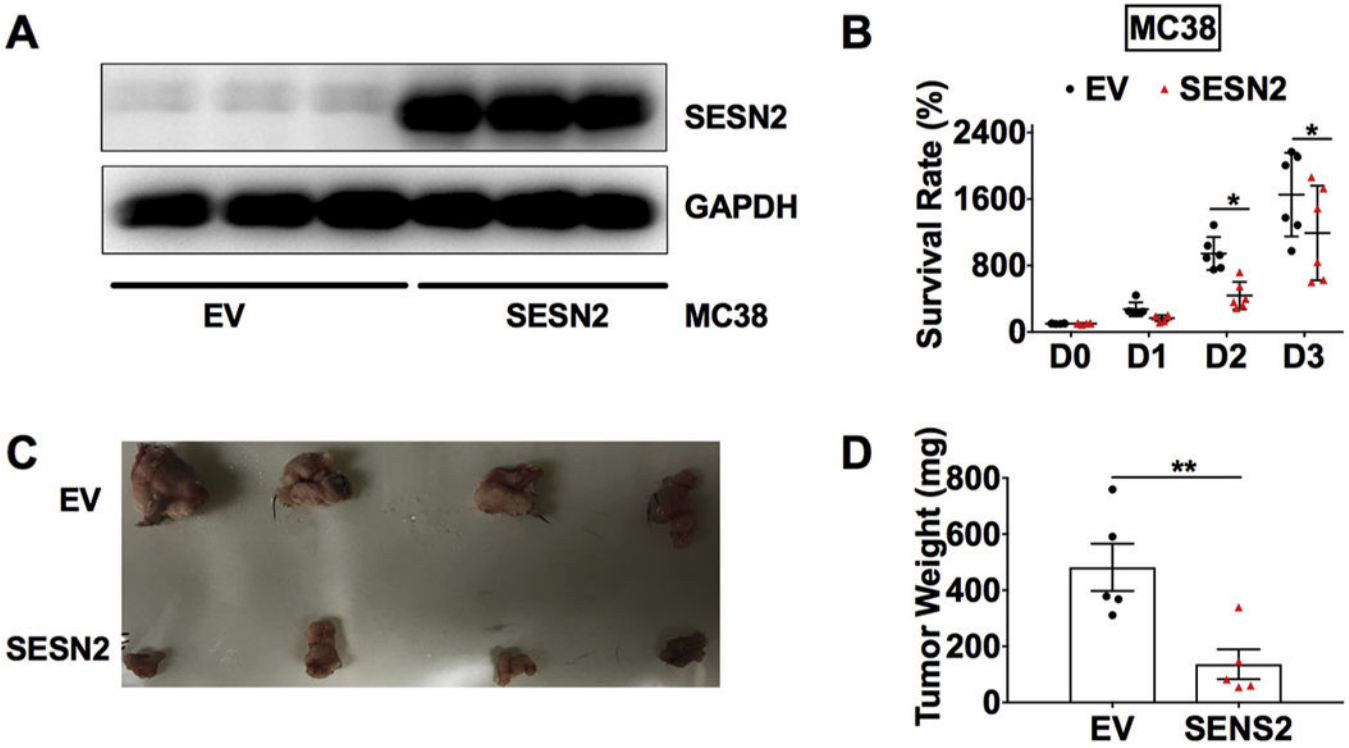

Fig. 6.

SESN2 overexpression reduces tumor cell growth and tumor size.

(**A**) Western blot analysis and (**B**) cell survival rate determined by MTT assay for MC38 cells stably expressed SESN2 or EV. (**C**) Representative images of MC38 tumors and (**D**) tumor weight at 14 days after subcutaneously injection of MC38 cells. $*p < .05$ and $**p < .01$.

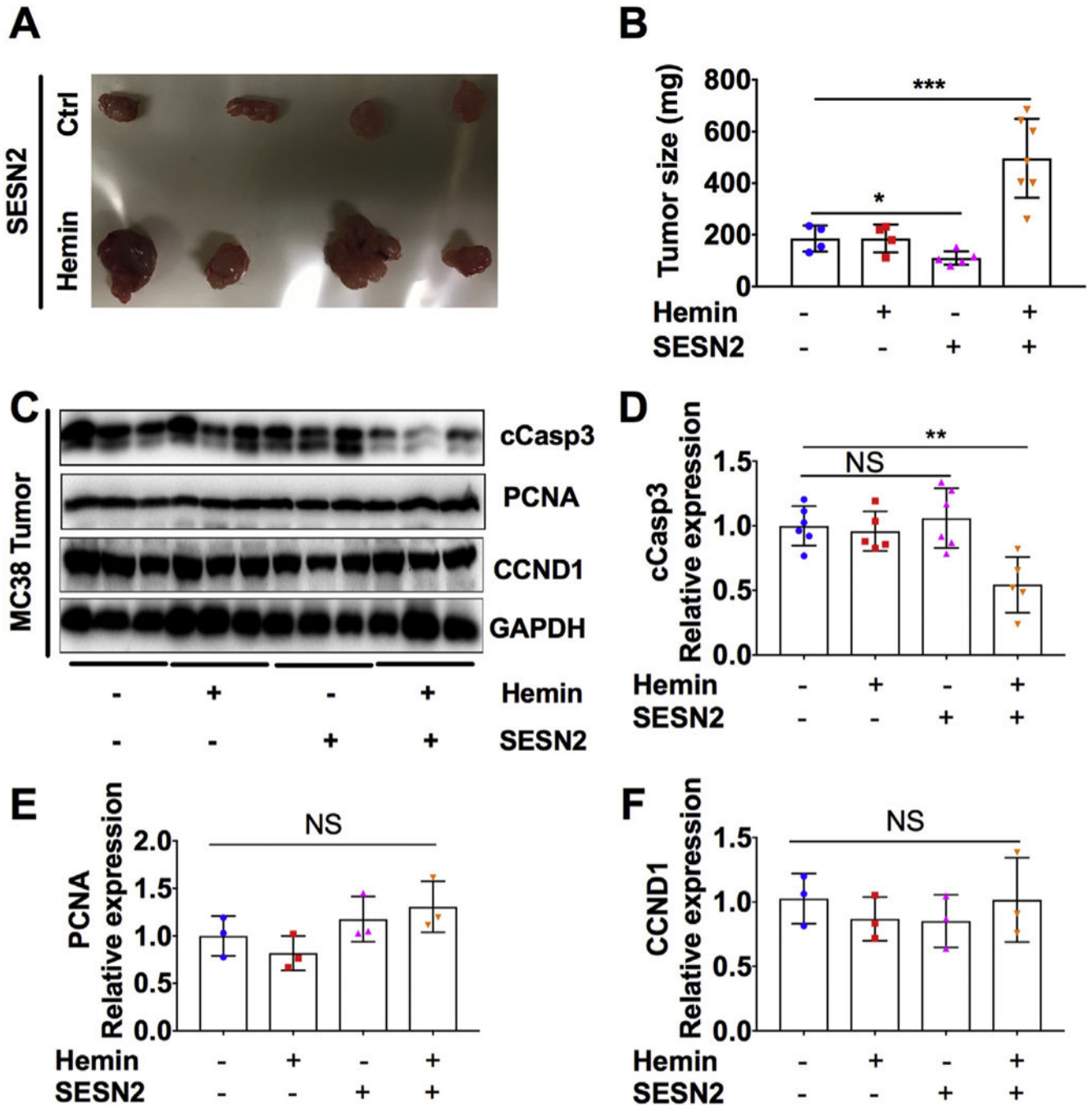

Fig. 7.

SESN2 overexpression increases tumor growth in the presence of hemin.

(**A**) Representative images of MC38 tumors and (**B**) tumor weight at 10 days after subcutaneously injection of MC38 cells treated with or without 100 μM hemin for overnight before injection. (**C–F**) Western blot analysis and quantification in MC38 tumors. $^{**}p < .01$ and $^{***}p < .001$. NS, not significant.

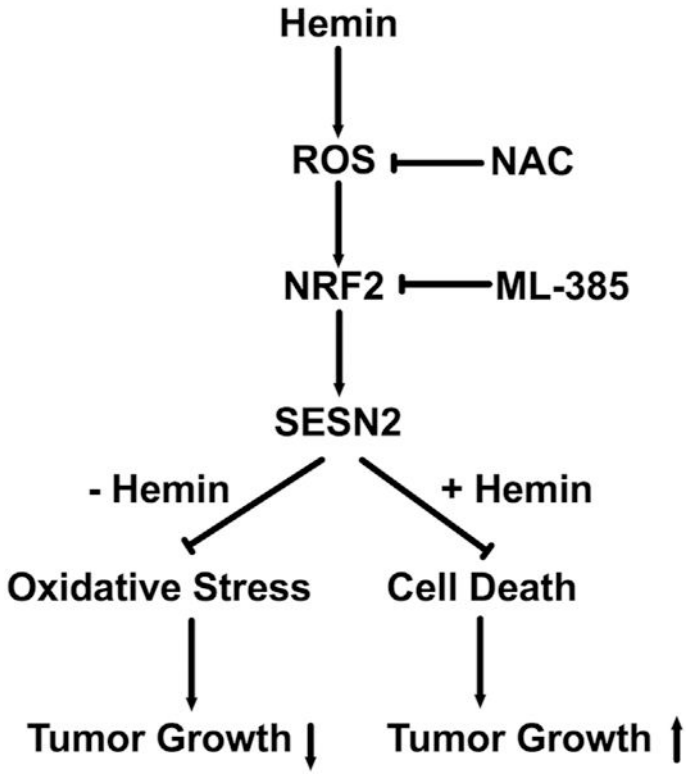

Fig. 8.

Proposed working model.

Hemin treatment induces ROS and activates NRF2, which lead to the enhanced expression of SESN2. The induction of SESN2 by hemin can be inhibited by the antioxidant NAC and NRF2 inhibitor ML385. In the absence of hemin, SESN2 overexpression leads to reduced oxidative stress and reduced tumor growth, whereas in the presence of hemin, SESN2 overexpression protects cell death and promotes tumor growth.