Sec23a Inhibits The Self-Renewal of Melanoma Cancer Stem Cells via Inactivation of ER-Phagy

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Abstract

Background

The genesis and developments of solid tumors, analogous to the renewal of healthy tissues, are driven by a subpopulation of dedicated stem cells, known as cancer stem cells (CSCs), that exhibit long-term clonal repopulation and self-renewal capacity. CSCs may regulate tumor initiation, growth, dormancy, metastasis, recurrence and chemoresistance. While autophagy has been proposed as a regulator of the stemness of CSCs, the underlying mechanisms requires further elucidation.

Methods

The subpopulation of CSCs in human melanoma cell line M14 was established by repetitive enrichments for cells that consistently display anchorage-independent spheroid growth. The stemness properties of the CSCs were confirmed *in vitro* by the expressions of stemness marker genes, the single-cell cloning assay and the serial spheroid formation assay. Subcutaneous tumor transplantation assay in BALB/c nude mice was performed to test the stemness properties of the CSCs *in vivo*. The autophagic activity in cells was confirmed by the protein level of LC3 and P62, mRFP-LC3B punta and cytoplasmic accumulation of autolysosomes. The morphology of ER was detected with transmission electron microscopy.

Results

In the present study, by employing a stable CSC cell line derived from human melanoma cell line M14, we show for the first time that Sec23a inhibits the self-renewal of melanoma CSCs via inactivation of ER-phagy. Mechanistically, inhibition of Sec23a reduces ER stress and consequently FAM134B-induced ER-phagy. Furthermore, TCGA data mining and analysis show that Sec23a is a favorable diagnostic and prognostic marker for human skin cutaneous melanoma (SKCM).

Conclusion

Herein, this study has elucidated a new mechanism underlying the regulation of autophagy on stemness, i.e. CSCs can exploit the SEC23A/ER-stress/FAM134B/ER-phagy axis for the self-renewal. The results provide new ideas for comprehensive exploration of the regulatory network of CSC self-renewal and new potential targets for CSCs-based therapy strategies for malignant tumors.

Background

Cutaneous melanoma is known for its high rate of metastasis and metastasis-related high mortality^{1–3}. The mean survival for metastatic melanoma is less than one year. The frequent sites of metastasis are

lung and the brain^{1,4,5}. The molecular mechanisms underlying melanoma metastasis awaits further elucidation. Accumulating research evidences have shown that metastasis is closely related to cancer stem cells (CSCs)^{6–8}. For many cancers, CSCs can be isolated from the bulk cell population and have clonal long-term repopulation and self-renewing capacity, distinct features that define CSCs. Although the involvement of CSCs in all aspects of human cancers have been postulated^{6,9}, the mechanisms governing the regulation of CSC self-renewal remain poorly defined.

Accumulating evidence indicates that autophagic activity may promote the stemness of CSCs to augment oncogenesis and metastatic progression^{10–12}. We recently reported that CSCs can exploit the autophagy-p53-Zeb1 axis for self-renewal, oncogenesis, and progression in lung cancer¹³. However, the mechanistic understanding underlying the regulation of CSC self-renewal by autophagy remains limited.

Autophagy is an evolutionarily conserved biological process of energy metabolism for the maintenance of homeostasis under nutrient deprivation or other stressful conditions^{14–16}. The endoplasmic reticulum (ER) is a dynamic organelle that regulates protein folding, Ca²⁺ buffering, and lipid and carbohydrate metabolism. Protein transportation defects may cause “ER stress” due to the accumulation of unfolded or misfolded proteins in the ER lumen. ER stress can activate ER-activated autophagy (ERAA) or ER-phagy^{14,17}. FAM134B is the ER-resident receptor that upon sensing ER-stress, will bind to autophagy modifiers LC3 and GABARAP which leads to the activation of ER-phagy¹⁸. Whereas regulation of CSC stemness by ER-phagy has been reported¹⁹, underlying mechanisms are elusive.

SEC23A is an important constituent of coat protein complex II (COPII) that is responsible for the transportation of secreted proteins from rough endoplasmic reticulum to Golgi apparatus^{20,21}. As a key regulator of the secretome^{22,23}, published studies, including ours, have been mainly focused on the secretome function of SEC23A^{24–26}. In our previous studies, we show that miR-200c augments melanoma metastasis by targeting Sec23a²⁵. We further show that S100A8 transported by SEC23A inhibits melanoma metastatic colonization via autocrine activation of autophagy in extravasated tumor cells, thus have identified for the first time the link between Sec23a and autophagy²⁷.

However, in this present study, using stable CSCs (M14-SE) derived from human melanoma cell line M14, we show for the first time that Sec23a inhibits the self-renewal of melanoma CSCs via inactivation of ER-phagy. Mechanistically, inhibition of Sec23a reduces ER stress and consequently FAM134B-induced ER-phagy. Furthermore, TCGA data mining and analysis show that Sec23a is a favorable diagnostic and prognostic marker for human skin cutaneous melanoma (SKCM).

Materials And Methods

Animals

All BALB/cA-nude mice used in the study were obtained from the core facility of Experimental Animal Centre in Chongqing Medical University. Animal studies were conducted in accordance with an approved protocol and with the institutional animal welfare guidelines of the Chongqing Medical University.

Cell culture

M14 melanoma cells were kindly provided by Dr. Robert Hoffman (University of California San Diego). Cells were cultured with DMEM supplemented with 1% Amphotericin B, 1% penicillin-streptomycin and 10% FBS. M14-SE cells were generated using the method we previously described, and cultured in the DMEM/F12 supplemented with 2% B27, 1% Amphotericin B and 1% penicillin-streptomycin, 20ng/ml EGF (Beyotime) and 20ng/ml FGF (Beyotime).

Lentivirus production

The sequence for the sh-RNAs targeting Sec23a was 5'-GGAAGCTACAAGAATGGTTGT-3'. The sequence for the sh-RNAs targeting FAM134B was 5'-GGAUCAAUUGAGAGUGAATT-3'. Sec23a overexpression (OE) plasmid pLVX-Puro-mRuby-Sec23a (Plasmid #36158) was provided by Addgene. The lentivirus particles of shSec23a, shFAM134B and Sec23a-OE were prepared by Sangon Biotech Co. The lentivirus particles of shAtg5 LV-ATG5-RNAi (9513-1) were purchased from GENECHM.

Autophagy inhibitors and activator

3-MA(M9281) was purchased from Sigma. Baf-A1(S1413) and Rapamycin (S1039) were purchased from Selleck.

Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNAs were isolated using Trizol (Takara, Japan) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara, Japan). RT-qPCR was performed using SYBR Green Real-time PCR Master Mix kit (Takara, Japan) according to the manufacturer's instructions. The following PCR condition was used on the Light Cycler: 39 cycles of 95°C for 30s, 95°C for 5s, followed by 60°C for 30s in a 10µl reaction volume. Relative expression was normalized to that of GAPDH internal control. The forward primer sequences and reverse primer sequences of Sec23a, Atg5, Bip, Pdi, Grp94, Edem, Atf4, FAM134B and GAPDH were AGTGGCGGAAGTCAGGATAC and GGCATTGGAAATCTGGAGTG; CTCTGCAGTGGCTGAGTGAA and TCAAT CTGTTGGCTGTGGGA; CAGTTGTTACTGTACCAGCCTA and CATTTAGGCCA GCAATAGTT CC; CCCTGCTGGTGGAAATTCTATG and GAAGAACTTGAGCGTAG GGTAC; TCTGAATTGATTGGCCAGTTTG and GGGTATCGTTGTTG TGTTTTGA; TCTTTGGCTACGACAACTACAT and CCAATGCATCA ACAAGAGTCAA; ATGGATTTGAAGGAGTTCGACT and AGAGATC ACAAGTGT CATCCAA; ACCCCATAGTTCATACTAGGC and CGTAA CAGAGGTTGGTGAGG; AGAAGGCTGGGGCTCATTTG and AGGG GCCATCCACAGTCTTC.

Western Blot (WB) analysis

WB was conducted as we previously reported²⁷. Cells were lysed in SDS lysis buffer (Beyotime, P0013G) containing 1% protease inhibitor PMSF (Beyotime, ST506). Extracted protein concentration was determined using the BCA protein assay kit (Beyotime, P0012S) and stored at -80°C. 20 µg of each protein sample were separated by electrophoresis with 12% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, IPVH00010). After blocking, the membranes were incubated with appropriate primary antibodies and secondary antibodies. The primary antibodies for WB were purchased from: SEC23A (Cell Signaling Technology®, #8162, 1:800), LC3B (Abcam, ab192890, 1:1000), P62 (Abcam, ab207305, 1:2000), ATG5 (Proteintech, 10181-2-AP, 1:1000), TUBULIN (Proteintech, 10068-1-AP, 1:2000), FAM134B (Proteintech, 21537-1-AP, 1:1000). The secondary antibodies of anti-Mouse (SA00001-1, 1:2000) and anti-Rabbit (SA00001-2, 1:2000) were purchased from Proteintech.

Autophagy flux analysis of LC3B puncta

Autophagy flux analysis of LC3B puncta was conducted as we previously reported²⁷. Adenovirus expressing mCherry-GFP-LC3B fusion protein (Ad-mCherry-GFP-LC3B, C3011) was purchased from Beyotime. Cells were plated in 6-well plates and allowed to reach 50%-70% confluence at the time of Ad-mCherry-GFP-LC3B transfection. Adenoviral infection was performed according to the manufacturer's instructions. The presence of mRFP-LC3B puncta indicated the autolysosomes in red fluorescent images.

Transmission electron microscopy (TEM) analysis of autolysosomes

Cells were harvested and centrifuged at 1200 rpm/min for 10 minutes. Cell pellet was fixed with 4% glutaraldehyde and 1% osmium tetroxide. Thereafter cell pellet was dehydrated in a graded series of alcohol and acetone and followed by embedment in Epon 816 (Electron Microscopy Sciences). Ultrathin sections were cut by a Leica ultramicrotome (Leica Microsystems) and stained with uranyl acetate and lead citrate. TEM was conducted by JEM-1400 Plus transmission electron microscope (JEOL Ltd).

TCGA database analysis

mRNA expression of Sec23a and in human skin cutaneous melanoma was analyzed by TCGA Research Network (<http://cancergenome.nih.gov>). The survival of patients with skin cutaneous melanoma was analyzed by OncoLnc (<http://www.oncolnc.org>).

Statistical analysis

All data were analyzed by Student's independent t-test of variance using GraphPad Prism software and presented as mean ± SEM. Differences were considered statistically significant when *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

Generation and characterization of M14 derived cancer stem cell line

The subpopulation of CSCs in M14 cell line was established by enriching the floating spheroids in the defined stem cell media, a method we have developed and previously described²⁸. The established CSCs cell line was named M14-SE and its control cell line was named M14-Parental (Fig. 1a). The enhancement of the stemness properties of the M14-SE cells was confirmed *in vitro* by: (1) the increased expression of stem markers including Aldh1, CD133, Nanog, Oct4 and Sox2 (Fig. 1b); enhanced self-renewal measured by (2) the single-cell cloning assay (Fig. 1c-e) as well as by (3) the serial spheroid formation assay (Fig. 1f). *In vivo*, 10⁴ cells of M14-Parental and M14-SE cells were injected subcutaneously into BALB/c nude mice. Tumors were exercised and weighed on day 16-day post tumor cell inoculation. The M14-Parental cells failed to develop into tumors in nude mice while the M14-SE cells successfully grow into evenly sized tumors in all inoculated mice (Fig. 1g-i). Collectively, both *in vitro* and *in vivo* experiments had confirmed that M14-SE cells represents the enrichment of the CSC component of the M14-parental cells, and were more oncogenic in comparison with the M14-Parental cells.

Sec23a inhibits the stemness of M14-SE cells

The expression level of Sec23a in M14-SE cells was significantly lower than that in M14-Parental cells (Fig. 2a-b). To investigate whether Sec23a influences the stemness of M14-SE cells, the stemness features were analyzed upon alteration of Sec23a expression. Stable Sec23a interference or overexpression was achieved by lentivirus infection, and confirmed by RT-qPCR (Fig. 2c) and Western blot (Fig. 2d), respectively. RT-qPCR analysis clearly showed that Sec23a expression is negatively correlated with the expression of a set of stemness-related genes (Aldh1, CD133, Nanog, Oct4 and Sox2) in M14-SE cells compared with the controls (Fig. 2e). *In vitro*, the single-cell cloning assay and the serial spheroid formation assay showed Sec23a interference significantly enhanced the self-renewal of M14-SE cells while Sec23a overexpression weakened it inversely (Fig. 2f-i). *In vivo*, subcutaneous tumor transplantation assay was performed in BALB/c nude mice (**Methods**). Tumors derived from M14-SE-shSec23a cells were significantly bigger and heavier than those of M14-SE-N.C. cells. On the contrary, tumors derived from M14-SE-Sec23a-OE cells were significantly smaller and lighter than those of M14-SE-vector cells (Fig. 2j-l). These results collectively show that Sec23a inhibits the stemness of M14-SE cells.

Autophagy promotes the stemness of M14-SE cells

While our prior work has shown for the first time the link between Sec23a and autophagy in regulation of M14 metastatic colonization²⁷, others have reported regulation of CSC stemness by autophagy^{29,30}. These observations prompted us to test whether autophagic activities differ in M14-parental and M14-SE cell. We thus compared the autophagic activity in the paired cell lines by: (1) the protein level of LC3 and P62 by western blot (WB), (2) mRFP-LC3B punta by immunofluorescence (IF), and (3) cytoplasmic accumulation of autolysosomes by transmission electron microscopy (TEM) analysis (**Methods**). We observed that autophagic activity was increased in M14-SE CSC cells compared with M14-parental cells,

as determined by the three assays (Fig. 3a-d). Furthermore, the level of autophagic activity is positively related to the stemness of M14-SE cells, measured by the single-cell cloning assay. Whereas autophagy inhibitors (3-MA and Baf-A1) treatment weakened the stemness, autophagy activator treatment augmented it (Fig. 3e-g). These observations suggest that autophagy augments the stemness of M14-SE cells.

To confirm this finding, autophagy was inhibited by stable Atg5 interference (Fig. 3h-i) and the efficiency was verified by RT-qPCR (Fig. 3j). *In vitro*, Atg5 interference significantly weakened the self-renewal of M14-SE cells as determined by the single-cell cloning assay and the serial spheroid formation assay (Fig. 3k-n). *In vivo*, subcutaneous tumor transplantation assay in BALB/c nude mice showed that tumors derived from M14-SE-shAtg5 cells were significantly smaller and lighter than those of M14-SE-N.C. cells (Fig. 3o-q). Based on these observations, we concluded that autophagy augments the stemness of M14-SE CSCs.

Sec23a inhibits the stemness of M14-SE cells via inhibition of autophagy

Thus far, we observed and confirmed that Sec23a inhibited the stemness of M14-SE cells (Fig. 2) and autophagy promoted the stemness of M14-SE cells (Fig. 3). We next evaluated whether Sec23a regulation of the stemness of M14-SE cells is dependent on autophagy using M14-SE-shSec23a and M14-SE-Sec23a-OE cell lines. WB analysis revealed a negative relationship between Sec23a expression and autophagy, i.e., autophagy was enhanced upon Sec23a interference and impaired with Sec23a overexpression in M14-SE cells (**Figure.4a**). The inhibitory effect of Sec23a on autophagy was confirmed by mRFP-LC3B punta analysis (Fig. 4b). The formation of autolysosomes was analyzed by TEM. Autolysosomes were markedly increased in M14-SE cells with Sec23a interference and were decreased in M14-SE cells with Sec23a overexpression (Fig. 4c-d). These results confirm that Sec23a inhibits autophagy in M14-SE cells.

We next examined the molecular relationship between autophagy and Sec23a. We inhibited autophagy by stable Atg5 interference in M14-SE-shSec23a cells (Fig. 4e-f) in which stemness of M14-SE cells was augmented (Fig. 2). Atg5 interference inhibited stemness-related gene expression in M14-SE-shSec23a-shAtg5 cells (Fig. 4g). This observation was confirmed by *in vitro* single-cell cloning assay and the serial spheroid formation assay (Fig. 4h-k). *In vivo*, tumors derived from M14-SE-shSec23a-shAtg5 cells were significantly smaller and lighter than those of M14-SE-shSec23a-N.C. cells (Fig. 4l-n). These observations show convincingly that Sec23a inhibits the stemness of M14-SE cells by inhibition of autophagy.

Sec23a inhibits autophagy in M14-SE cells via inhibition of ER stress

Sec23a is responsible for the transport of secretory proteins from ER to Golgi apparatus. It was reported that Sec23a could regulate ER stress^{22,31}. To investigate the specific mechanism by which Sec23a inhibits autophagy, we first measured the level of the ER stress in M14-SE cells with Sec23a interference

(M14-SE-shSec23a cells) or overexpression (M14-SE-Sec23a-OE cells) by assessing the expression of five ER stress maker genes (Fig. 5a). Sec23a expression was inversely related to the expression of ER stress genes (Fig. 5a), i.e., Sec23a appeared to inhibit ER stress. TEM showed the ER in M14-SE-shSec23a cells presented a typical stress response (Fig. 5b), consistent with the molecular findings (Fig. 5a).

Since ER stress can induce ER-phagy, a specific form of autophagy^{32,33}, we next determined whether inhibition of ER-stress by Sec23a leads to reduction of ER-phagy. The expression of FAM134B, an important ER-phagy receptor protein, was inversely related to Sec23a expression level in M14-SE cells (Fig. 5c-d). To examine whether FAM134B mediates Sec23a inhibition of ER-phagy, stable FAM134B interference was achieved by lentivirus infection in M14-SE-shSec23a cells (Fig. 5e-f). WB analysis of P62 and LC3 lipidation showed that FAM134B interference significantly inhibited autophagy activity (Fig. 5f). The effect of FAM134B on autophagic activity was verified by mRFP-LC3B puncta and autolysosomes formation (Fig. 5g-i). This set of results show that elevation of ER stress upon Sec23a interference, sensed by FAM134B will lead to augmented autophagic/ER-phagic activity.

FAM134B promotes the stemness of M14-SE cells

To confirm that ER stress/ER-phagy regulates the stemness of M14-SE cells, we assayed the expressions of stemness related genes upon FAM134B interference. FAM134B interference significantly inhibited the stemness-related genes in M14-SE-shSec23a cells (Fig. 6a). *In vitro*, the single-cell cloning assay and the serial spheroid formation assay showed that FAM134B interference significantly hindered the self-renewal capability of M14-SE-shSec23a cells (Fig. 6b-e). *In vivo*, tumors derived from M14-SE-shSec23a-N.C. cells were significantly bigger and heavier than those of M14-SE-shSec23a-shFAM134B cells (Fig. 6f-h).

Sec23a is a favorable diagnostic and prognostic marker for SKCM

To assess the clinical relevance of our novel finding that Sec23a inhibits the self-renewal of melanoma CSCs, we performed clinical data analysis using the TCGA (The Cancer Genome Atlas). Kaplan-Meier plots analysis revealed that skin cutaneous melanoma (SKCM) patients with high Sec23a expression levels had significantly better overall survivals in comparison with patients with low Sec23a expression level (Fig. 6i). Furthermore, Sec23a expression level was significantly lower in SKCM patients with advanced primary tumors and regional lymph node metastasis in comparison with SKCM patients of early stages (Fig. 6j-k).

Discussion

Human skin cutaneous melanoma (SKCM) is characterized by its high rate of metastasis and mortality¹. More effective clinical management of SKCM requires improved understanding of the molecular mechanisms underlying melanoma stem cell self-renewal which may play a pivotal role in melanoma metastasis^{34,35}. Accumulating evidence have shown augmentation of CSC self-renewal by autophagy³⁶⁻

³⁸. In a recent study, we reported for the first time that autophagy augments the stemness of lung CSCs by degrading ubiquitinated TP53. Further, Zeb1 is required for TP53 regulation of CSC self-renewal¹³. The present study has made a set of novel findings to elucidate a new CSC self-renewal mechanism that has not been described before, i.e., Sec23a inhibits the stemness of melanoma CSCs by inactivation of ER-stress induced ER-phagy.

Although a positive correlation between autophagic activities and the stemness of CSCs has been reported in breast, pancreatic, liver, osteosarcoma, ovarian and glioblastoma CSCs^{39–42}, the underlying mechanisms remain elusive. In the present study, we show that mechanistically, inhibition of Sec23a promotes ER stress and consequently FAM134B-conducted ER-phagy. Activation of autophagy by Endoplasmic reticulum (ER) stress is an evolutionarily conserved mechanism for the maintenance of cellular homeostasis^{43,44}. The ER stress-mediated autophagy is characterized by the generation of autophagosomes that include worn-out proteins, protein aggregates, and damaged organelles⁴⁵. Signaling pathways of IRE1 α , PERK, ATF6 and Ca²⁺ are necessary for the activation of ER stress-mediated autophagy^{46–48}, while the receptor-mediated ER-phagy that degrades the ER requires FAM134B^{49–51}. FAM134B is an ER-resident receptor which contains a conserved putative LC3-interacting region (LIR motif). FAM134B binds to autophagy modifiers LC3 and GABARAP, which in turn facilitates ER degradation by ER-phagy¹⁸. We have revealed for the first time here that ER-phagy, activated upon sensing ER-stress by FAM134B, augments the stemness of melanoma CSCs.

Since the first report on the inhibitory role of Sec23a in cancer metastasis²⁴, mechanistic investigations have been focused on remodeling of tumor microenvironment by Sec23a-regulated cancer cell secretome at the site of distant metastasis^{25,52}. Consistent with the literature, we show that Sec23a inhibits metastatic colonization efficiency by changing the secretome to activate autophagic activity in melanoma cells. It is achieved via the Sec23a-S1008A-Beclin1 axis²⁷. In a recent report, SEC23B, a homolog protein of the SEC23 family, can activate autophagy under nutrient deprivation condition⁵³. However, secretome-independent role of Sec23a in regulation of tumor metastasis has not been reported. In addition, the connection between Sec23a and CSC self-renewal has not been established and reported prior to our present study. By employing the stable CSC cell line derived from human melanoma cell line M14 (ME14-SE), we show for the first time that Sec23a inhibits the self-renewal of melanoma CSCs via inactivation of ER-phagy. We have elucidated a novel CSC-based mechanism underlying the high metastatic rate of SKCM that has important clinical relevance. What's more, TCGA data mining and analysis show that Sec23a, the regulator of the ER-stress/FAM134B/ER-phagy axis, is a favorable diagnostic and prognostic marker for SKCM. Future studies using clinical cohorts are required to validate the diagnostic and prognostic importance of Sec23a in SKMC.

In summary, this study has elucidated a new mechanism underlying the regulation of autophagy on stemness, i.e. CSCs can exploit the SEC23A/ER-stress/FAM134B/ER-phagy axis for the self-renewal. The results provide new ideas for comprehensive exploration of the regulatory network of CSC self-renewal and new potential targets for CSCs-based therapy strategies for malignant tumors.

Conclusions

In conclusion, our research findings reveal for the first time that Sec23a inhibits the self-renewal of CSCs via inactivation of ER-phagy. The expression level of Sec23a in CSCs is significantly lower than that in parental tumor cells. Stable Sec23a interference or overexpression respectively promotes or inhibits the self-renewal of CSCs *in vitro* and *in vivo*. Sec23a is responsible for the transportation of secreted proteins from rough endoplasmic reticulum to Golgi apparatus. Inhibition of Sec23a significantly gives rise to ER-stress and promotes the expression of FAM134B, and eventually enhances the ER-phagy which promotes the self-renewal of CSCs. FAM134B plays a pivotal role in ER stress-induced ER-phagy. And this present study also reveals for the first time that FAM134B promotes the self-renewal of CSCs. Stable FAM134B interference significantly impairs the self-renewal of CSCs *in vitro* and *in vivo*. The clinical data analysis with TCGA database shows that higher Sec23a expression level appears to be protective factors and favorable diagnostic (TNM staging) and prognostic (overall survival) marker for SKCM patients. The research findings improve our understandings of self-renewal regulations of CSCs and provide potential targets for CSCs-based therapy strategies.

Abbreviations

CSCs
cancer stem cells; ER:endoplasmic reticulum; Sec23a:Sec23 homolog A; FAM134B:RETREG1, reticulophagy regulator 1; COPII:coat protein complex II; SKCM:skin cutaneous melanoma; OE:overexpression; TEM:transmission electron microscopy; IF:immunofluorescence; TCGA:The Cancer Genome Atlas.

Declarations

Ethics approval and consent to participate

Animal studies were conducted in accordance with an approved protocol and with the institutional animal welfare guidelines of the Chongqing Medical University.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zhiwei Sun performed the experiments and data analysis and wrote this manuscript. Doudou Liu, Bin Zeng, Qiting Zhao, Xiaoshuang Li and Hao Chen participated in the conduction of this study. H.Rosie Xing and Jianyu Wang designed this study, oversaw the execution of this study, and contributed to the revision of this manuscript. All authors read and approved the final manuscript.

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Figures

Figure.1

Figure 1

Generation and characterization of M14 derived cancer stem cell line. (a). The morphology of M14-Parental and M14-SE cells, bar=120μm. (b). Analysis of mRNA expression of Aldh1, CD133, Nanog, Oct4 and Sox2. The GAPDH expression was used as a reference control. (c-e). The clonogenic spheroid formation rate, morphology and average spheroid diameter of M14-Parental and M14-SE cells in the single-cell cloning assay. (f). The serial spheroid formation assay of M14-Parental and M14-SE cells, 1st means single-cell suspensions were plated at 1000 cells/well, 2nd means spheroid cultures were then collected from 1st and single-cell suspensions were prepared for setting up the second round of the assay at 1000 cells/well, And so on, the 3rd means 1000 cells come from the 2nd, bar=200μm. (g). Tumors resected from nude mice inoculated with M14-Parental and M14-SE cells, respectively, bar=1cm. (h and i). Tumor volume and weight in nude mice to which M14-Parental and M14-SE cells were injected respectively. **P<0.01, ***P<0.001.

Figure.2

Figure 2

Sec23a inhibits the stemness of M14-SE cells. (a and b). RT-qPCR and Western blot were performed to detect Sec23a expression in M14-Parental and M14-SE cells. (c and d). RT-qPCR and Western blot were performed to confirm gene manipulation of Sec23a expression in M14-SE cells. (e). Analysis of mRNA expression of Aldh1, CD133, Nanog, Oct4 and Sox2 in M14-SE cells with Sec23a interference or overexpression. The GAPDH expression was used as a reference control. (f-h). The clonogenic spheroid formation rate, morphology and average spheroid diameter of M14-SE cells with Sec23a interference or overexpression in the single-cell cloning assay. (i). The serial spheroid formation assay of M14-SE cells with Sec23a interference or overexpression, bar=200μm. (j). Tumors resected from nude mice inoculated with M14-SE cells with Sec23a interference or overexpression, bar=1cm. (k and l). Tumor volume and weight in nude mice to which M14-SE cells with Sec23a interference or overexpression were injected. *P<0.05, **P<0.01, ***P<0.001.

Figure.3

Figure 3

Autophagy promotes the stemness of M14-SE cells. (a). Western blot analysis for LC3I/II and P62 in M14-Parental and M14-SE cells. (b). M14-Parental and M14-SE cells expressed mRFP-LC3B fusion protein via adenovirus infection. Representatives of LC3B-positive puncta images were shown, bar=20μm. (c). Transmission electron microscopy of M14-Parental and M14-SE cells. Autolysosomes indicated by arrowheads, bar=5μm. (d). Mean number of detectable autolysosomes in each tumor cell, counted on

transmission electron microscopy images. (e-g). The clonogenic spheroid formation rate, morphology and average spheroid diameter of M14-SE cells treated with DMSO, 3-MA, Baf-A1 and Rapa in the single-cell cloning assay. (h and i). RT-qPCR and Western blot were performed to confirm Atg5 interference in M14-SE cells. (j). Analysis of mRNA expression of Aldh1, CD133, Nanog, Oct4 and Sox2 in M14-SE cells with Atg5 interference. (k-m). The clonogenic spheroid formation rate, morphology and average spheroid diameter of M14-SE cells with Atg5 interference in the single-cell cloning assay. (n). The serial spheroid formation assay of M14-SE cells with Atg5 interference, bar=200 μ m. (o). Tumors resected from nude mice inoculated with M14-SE cells with Atg5 interference, bar=1cm. (p and q). Tumor volume and weight in nude mice to which M14-SE cells with Atg5 interference were injected. *P<0.05, **P<0.01, ***P<0.001.

Figure.4

Figure 4

Sec23a inhibits the stemness of M14-SE cells via inhibition of autophagy. (a). Western blot analysis for LC3I/II and P62 in M14-SE cells with Sec23a interference or overexpression. (b). Representatives of LC3B-positive puncta images were shown in M14-SE cells with Sec23a interference or overexpression, bar=20μm. (c). Transmission electron microscopy of M14-SE cells with Sec23a interference or overexpression. Autolysosomes indicated by arrowheads, bar=5μm. (d). Mean number of detectable

autolysosomes in each tumor cell, counted on transmission electron microscopy images. (e and f). RT-qPCR and Western blot were performed to confirm Atg5 interference in M14-SE-shSec23a cells. (g). Analysis of mRNA expression of Aldh1, CD133, Nanog, Oct4 and Sox2 in M14-SE-shSec23a cells with Atg5 interference. (h-j). The clonogenic spheroid formation rate, morphology and average spheroid diameter of M14-SE-shSec23a cells with Atg5 interference in the single-cell cloning assay. (k). The serial spheroid formation assay of M14-SE-shSec23a cells with Atg5 interference, bar=200 μ m. (l). Tumors resected from nude mice inoculated with M14-SE-shSec23a cells with Atg5 interference, bar=1cm. (m and n). Tumor volume and weight in nude mice to which M14-SE-shSec23a cells with Atg5 interference were injected. **P<0.01, ***P<0.001.

Figure.5

Figure 5

Sec23a inhibits autophagy in M14-SE cells via inhibition of ER stress. (a). Analysis of mRNA expression of ER stress marker genes in M14-SE cells with Sec23a interference or overexpression. (b). The morphology of ER in M14-SE cells with Sec23a interference or overexpression under transmission electron microscopy, bar=5μm. (c and d). RT-qPCR and Western blot were performed to detect FAM134B expression in M14-SE cells with Sec23a interference or overexpression. (e). RT-qPCR was performed to confirm FAM134B interference in M14-SE-shSec23a cells. (f). Western blot analysis for FAM134B, LC3I/II

and P62 in M14-SE-shSec23a cells with FAM134B interference. (g). Representatives of LC3B-positive puncta images were shown in M14-SE-shSec23a cells with FAM134B interference, bar=20μm. (h). Transmission electron microscopy of M14-SE-shSec23a cells with FAM134B interference. Autolysosomes indicated by arrowheads, bar=5μm. (i). Mean number of detectable autolysosomes in each tumor cell, counted on transmission electron microscopy images. *P<0.05, **P<0.01, ***P<0.001.

Figure.6

Figure 6

FAM134B promotes the stemness of M14-SE cells and Sec23a is a favorable diagnostic and prognostic marker for SKCM. (a). Analysis of mRNA expression of Aldh1, CD133, Nanog, Oct4 and Sox2 in M14-SE-shSec23a cells with FAM134B interference. (b-d). The clonogenic spheroid formation rate, morphology

and average spheroid diameter of M14-SE-shSec23a cells with FAM134B interference in the single-cell cloning assay. (e). The serial spheroid formation assay of M14-SE-shSec23a cells with FAM134B interference, bar=200 μ m. (f). Tumors resected from nude mice inoculated with M14-SE-shSec23a cells with FAM134B interference, bar=1 cm. (g and h). Tumor volume and weight in nude mice to which M14-SE-shSec23a cells with FAM134B interference were injected. (i). Low Sec23a expression levels indicated poor overall survivals for SKCM patients using Kaplan-Meier Plots analysis. (j and k) Correlation between Sec23a expression and tumor stages in SKCM patients using the TCGA database. *P<0.05, **P<0.01, ***P<0.001.