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Sec23a mediates miR-200c augmented oligometastatic to polymetastatic progression

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ABSTRACT

Background: Cancer treatment is based on tumor staging. Curative intent is only applied to localized tumors. Recent studies show that oligometastatic patients who have limited number of metastases may benefit from metastasis-directed local treatments to achieve long-term survival. However, mechanisms underlying oligometastatic to polymetastatic progression remains elusive.
Methods: The effects of miR-200c and Sec23a on tumor metastasis were verified both *in vitro* and *in vivo*. The secretome changes were detected by mass spectrometry.
Findings: We established a pair of homologous lung-metastasis derived oligometastatic and polymetastatic cell lines from human melanoma cancer cell line M14. Using the two cell lines, we have identified *Sec23a*, a gene target of miR-200c, suppresses miR-200c augmented oligometastatic to polymetastatic progression *via* its secretome. Firstly, miR-200c over-expression and *Sec23a* interference accelerated oligometastatic to polymetatic progression. Secondly, *Sec23a* functions downstream of miR-200c. Thirdly, mass spectrometric analysis of the secretory protein profile suggests that *Sec23a*-dependent secretome may impact metastatic colonization by modifying tumor microenvironment. Fourthly, the survival analysis using The Cancer Genome Atlas database shows *Sec23a* as a favorable prognostic marker for skin cutaneous melanoma, supporting the clinical relevance of our findings.
Interpretation: The finding that Sec23a is a suppressor of oligometastatic to polymetastatic progression has clinical implications. First, it provides a new theoretical framework for the development of treatments that prevent oligometastasis to polymetastasis. Second, Sec23a may be used as a favorable prognostic marker for the selection of patients with stable oligometastatic disease for oligometastasis-based local therapies of curative intent.
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1. Introduction

Cancer treatment is based on tumor staging. Curative intent is only applied to early-stage localized tumors. Recent studies show that in contrast to widespread polymetastases, oligometastatic patients who have limited number or spread of metastases may benefit from metastasis-directed local treatments [1–4]. Surgical resection has been proven to have curative effects on limited metastases in lung [5,6], liver [7,8] and breast [9,10]. Moreover, treatment of limited metastases with SBRT (stereotactic body radio therapy) has achieved long-term survival [11,12]. However, mechanisms underlying oligometastatic to polymetastatic progression remain elusive. Identification of predictors or mediators of progression will improve patient selection for metastasis-directed therapy, or for the development of novel therapies that prevent oligometastatic to polymetastatic progression.

MiRNAs are a class of non-coding single-stranded RNA molecules of about 22 nucleotides in length encoded by endogenous genes that are involved in in normal and pathological processes [13,14]. Metastasis is

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Research in context

Evidence before this study

Cancer treatment is based on tumor staging. Curative intent is only applied to early-stage localized tumors. Recent studies show that oligometastatic patients who have limited number or spread of metastases may benefit from metastasis-directed local treatments and achieve long-term survival. However, our understanding about mechanisms underlying oligometastatic to polymetastatic progression remains limited.

Added value of this study

In this study, we characterized the metastasis promoting effect of miR-200c in the context of oligometastatic to polymetastatic progression, providing more experimental evidence for the context-dependency of the role of miR-200c in tumor metastasis. We have demonstrated for the first time that *Sec23a*, a gene target of miR-200c, suppresses miR-200c augmented oligometastatic to polymetastatic progression *via* its secrotome function. Further, survival analysis using The Cancer Genome Atlas (TCGA) database showed *Sec23a* as a favorable prognostic marker for skin cutaneous melanoma, supporting the clinical relevance of our findings. In summary, our study provided new mechanistic understanding on oligometastatic to polymetastatic progression.

Implications of all the available evidence

The findings have provided a new theoretical framework for future research to explore the translational potential for using Sec23a as a biomarker for selection of oligometastatic patients for therapies of curative intent. Since many secreted proteins affecting tumor progression can be regulated by Sec23a, whether Sec23a can be a therapeutic target for the development of oligometastasis-based therapies merits further investigation.

a multi-step process and all steps are subjected to the regulation of miRNAs [15,16]. MiR-200c can exert both inhibitory and stimulatory effects on tumor metastasis which are context-dependent. In primary tumors, miR-200c suppresses invasion by inhibiting epithelial-to-mesenchymal transition (EMT) *via* Zeb1/Zeb2-E-cadherin axis [17,18]. In contrast, miR-200c promotes tumor metastasis at the step of colonization at the distant site [19]. However, the mechanisms underlying the metastasis-promoting effect of miR-200c have not been well elucidated.

Establishment of favorable tumor microenvironment at a distant site can be achieved in part by secretome which consists of proteins secreted by tumor cells [20]. Coat protein complex II(COPII) is a type of vesicle coat protein that transports proteins from the rough endoplasmic reticulum to the Golgi apparatus [21,22]. The COP II pathway is classified as the classical secretory protein pathway. The COPII complex, consists of large protein subcomplexes that include the Sec23p/Sec24p heterodimer and the Sec13p/Sec31p heterotetramer [21,23,24]. Sec23 homolog A(SEC23A), encoded by the *Sec23a* gene. *Sec23a* is a member of the SEC23 subfamily of the SEC23/SEC24 family that is responsible for the assembling of COPII [21,25]. The expression of *Sec23a* has been reported to be lower in the advanced stage tumors than that in the early stage tumors [19,26].

In our previous study, miRNA-microarray profiling had identified *Sec23a* gene as a gene target of miR-200c [27,28]. While the metastasis-promoting activity of miR-200c was reported to be mediated by Sec23a [19], the function of miR-200c and *Sec23a* in the context of oligometastatic to polymetastatic progression has not been characterized. In the present study, we identified and characterized the regulatory effects of miR-200c-*Sec23a*-secreted proteins axis in oligometastatic to polymetastatic progression at the step of colonization. We demonstrated for the first time that Sec23a is a suppressor of oligometastatic to polymetastatic progression, providing a new theoretical framework for the development of treatments that prevent oligometastasis to polymetastasis.

2. Materials and methods

2.1. Cell culture

GFP-labeled M14 were kindly provided by Dr. Robert Hoffman (University of California San Diego). M14, OL, POL, OL-N.C., OL-sh*Sec23a*, OL-miR-200c-OE, OL-miR-200c-OE-vector and OL-miR-200c-OE-*Sec23a*-OE were maintained in DMEM high glucose supplemented (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (ExCell Bio, Shanghai, China), and incubated at 37 °C in humidified air containing 5% CO_2.

2.2. Transwell migration and invasion assays

Transwell inserts (8 μm pore size, BD Falcon) were used to perform cell invasion assay with Matrigel (100 μl, 1:8 dilution in serum free medium, BD Biosciences) and migration assay without Matrigel. 300 μl serum-free medium with 5×10^4 cells was seeded into the upper chamber, while 800 μl medium with 20% FBS as chemo-attractant was added into the lower chamber. After 24 h of incubation, cells were removed from the upper surface of the porus membrane with a cotton swab, followed by fixation of cells migrated and invaded to the lower surface of the membrane with 70% ethanol for 1 h and staining with crystal violet for 15 min. Stained cells were counted under light microscopy and 10 random fields from three replicate Transwells were counted. The number of migrated and invaded cells was presented as number of cells counted per field of the porous membrane.

2.3. Cell colony formation assay

A total of 200 cells in 1 ml DMEM containing 0.2% agar (Solarbio Science & Technology Co., Beijing, China) and 10% FBS were plated per well into 6-well plates coated with 1 ml DMEM containing 0.5% agar and 10% FBS. After 1 week, colonies were stained with crystal violet and colonies consist of no less less 50 cells counted. The experiment was performed in triplicate and repeated three times.

2.4. Animal experiments

All mice used in the study were obtained from the core facility of Experimental Animal Centre in Chongqing Medical University. All animal work was conducted in accordance with an approved protocol and carried out in accordance with the institutional animal welfare guidelines of the Chongqing Medical University. 5×10^5 tumor cells were transplanted into each male NOD/SCID mouse *via* tail intravenous injection. Mice were weighted every 3 days and dissected after 27 days post tumor cell injection. Sellstrom Z87 fluorescence goggles and an LDP 470 nm bright blue flashlight were used for examining the metastases in the mice. Metastatic nodules in the lungs were counted at the time of sacrifice and confirmed by H&E staining.

2.5. CCK-8 Cell proliferation assay

Cell proliferation was determined with the cell counting kit (CCK)-8 assay (Beyotime, Shanghai, China). Cells were plated in 96-well plates at a density of 2×10^3 cells per well. The absorbance of each well at 450 nm was measured with an enzyme-linked immunosorbent assay reader (Tecan M200 PRO NanoQuant). Cell proliferation activity was measured

for successive 6 days. Cell doubling time (DT) was calculated with the eq. DT = Δt × lg2/(lgNt-lgN0).

2.6. Lentivirus-mediated stable silencing of Sec23a, over-expression of miR-200c and overexpression of Sec23a

The sequence for the control short hairpin RNA was 5′-TTCTCCGAA CGTGTCACGT-3′. The sequence for the sh-RNAs targeting *Sec23a* was 5′-GGAAGCTACAAGAATGGTTGT-3′. The lentivirus particles of N.C., sh-*Sec23a* and miR-200c overexpression (OE) were purchased from Sangon Biotech Co. (Shanghai, China). *Sec23a* overexpression plasmid pLVX-Puro-mRuby-*Sec23a* (Plasmid #36158) was provided by Addgene (Massachusetts, USA) and the lentivirus particles were prepared by Sangon Biotech Co. (Shanghai, China). Cells were infected with a multiplicity of infection (MOI) of 50 according to the manufacturer's protocol. Stable OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE cells were purified by flow fluorescence sorting. After 72 h of infection, RT-qPCR and western blotting assays were performed to measure the infection efficiency.

2.7. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted with Trizol (TaKaRa, Dalian, China). 2 μg of total RNA was subjected to reverse transcription with PrimeScript RT Master Mix (TaKaRa, Dalian, China). For the reverse transcription of miRNA, Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Dalian, China) was used. RT-qPCR was performed using a SYBR Green Real-time PCR Master Mix kit (TaKaRa, Dalian, China) under the following condition: initial pre-incubation at 95 °C for 30s, followed by 39 cycles at 95 °C for 5 s and 60 °C for 30s. The relative mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. The forward primer sequence of miR-200c was GCTAATACTGCCGGGTAATGATG and the forward primer sequence of U6 was GCTTCGGCAGCACACA TACTAAAAT. The forward primer sequence and reverse primer sequence of *Sec23a* were AGTGGCGGAAGTCAGGATAC and GGCATTGGA AATCTGGAGTG. The forward primer sequence and reverse primer sequence of GAPDH were AGAAGGCTGGGGCTCATTTG and AGGGGCCAT CCACAGTCTTC.

3. Western blotting analysis

Cells were lysed in 200 μl SDS lysis buffer (Beyotime, Shanghai, China) with 1% PMSF (Beyotime, Shanghai, China) and boiled at 100 °C for 15 min. 40 μg of each protein sample was separated by electrophoresis with 12% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, CA, USA). Thereafter, according to the manufacturer's instruction, the proteins were incubated with appropriate primary antibodies and secondary antibodies. Primary antibodies of SEC23A and the loading control (TUBULIN) were purchased from Cell Signaling Technology (Massachusetts, USA). Primary antibodies of Thrombospondin-1 and CXCL4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies of Transferrin, S100A8 and CYB5R3 were purchased from R&D Systems (Minnesota, USA). Results were analyzed by ImageJ version 1.47 (National Institutes of Health, Maryland, USA). Three independent experiments were done for statistical analysis.

4. Secretory protein profile analysis

Conditioned media from different cell lines were concentrated with a vacuum concentrator (Thermo Scientific Savant DNA120, Thermo Fisher Scientific, USA) and centrifuged in 3-kDa ultracentrifuge tube (Merck Millipore, Massachusetts, USA), followed by protein quantization and SDS-PAGE electrophoresis. Thereafter, protein samples were subjected to reductive alkylation, enzymatic hydrolysis and labeled with Tandem Mass Tags (TMT) 10-plex Isobaric Label Reagent Set (Thermo Fisher Scientific, Massachusetts, USA). Reversed phase liquid chromatography (RPLC) analysis was performed on the Agilent 1200 HPLC System (Agilent, CA, USA). Online Nano-RPLC analysis was conducted on the Easy-nLC 1000 System (Thermo Fisher Scientific, Massachusetts, USA), and samples were loaded onto NanoLC trap column (PepMap100, NanoViper, Thermofisher Dionex). Data acquisition was performed with Q-Exactive System (Thermo Fisher Scientific, Massachusetts, USA) fitted with a Nanospray. The spectral data files were tanalyzed using the SEQUEST algorithm available in Proteome Discoverer 1.4 software (Thermo Fisher Scientific, Massachusetts, USA).

4.1. Statistical analysis

All experiments were performed at least three times for statistical analysis. Quantitative results were shown as mean ± SEM (standard error of the mean). Data were analyzed with GraphPad Prism version 5.0 (GraphPad Software Inc., CA, USA) by two-tailed Student's *t*-test. Images were globally adjusted with Photoshop version 11.0.1 (Adobe Systems Inc., CA, USA). $P < .05$ was considered significant statistically and was marked with an asterisk. $P < .01$ and $P < .01$ were considered highly statistically significant and were marked with double asterisks and triple asterisks respectively.

Ethics statement

All mice used in the study were obtained from the core facility of Experimental Animal Centre in Chongqing Medical University. All animal work was conducted in accordance with an approved protocol and carried out in accordance with the institutional animal welfare guidelines of the Chongqing Medical University.

5. Results

5.1. Characterization of oligometastatic and polymetastatic cells in vitro and in vivo

By isolation of M14 cells from the lung of mice exhibited oligometastasis or polymetastasis respectively *in vivo*, followed by three rounds of *in vivo* validation, a set of paired stable lung-derived oligometastatic cell model (OL) and the homologous polymetastatic cell model (POL) from human melanoma cancer cell line M14 were established [29]. The migration and invasion capacities of the OL and POL cells were examined using the Transwell chambers. Compard with OL cells, POL cells exhibited significantly elevated migration and invasion ability *in vitro* (Fig. 1a-d, $P < .001$). Colony formation assay was performed to compare the metastatic colonization capacities of the OL and POL cells (Methods). POL cells were more capable of metastatic colonization than the OL cells *in vitro* (Fig. 1e-f, $P < .01$). Tail vein injection of tumor cells to the immunodeficient NOD/SCID mice was performed to access metastatic colonization efficiency *in vivo* (Methods). Mice received POL cells developed much more GFP-labeled macroscopic metastatic foci in the lungs than that of the OL cells, as evident by whole lung fluorescent imaging (Fig. 1g), H&E staining and quantitative H&E analysis (Fig. 1h-i, $P < .001$). Tail vein injection of POL cells also resulted in the development of more metastatic foci in the thoracic cavities (Supplementary Fig. 1a) and suffered from more severe weight loss (Supplementary Fig. 1b) than that of OL cell injected mice. These observations demonstrate that POL cells have higher metastatic capability than the OL cells both *in vitro* and *in vivo*.

6. MiR-200c over-expression and *Sec23a* interference augmented metastasis

While POL and OL cells differed greatly in the capability of invasion and metastasis (Fig. 1), they had comparable doubling time for cell proliferation (Methods, Fig. 2a, $P > .05$), confirming the differences in cell

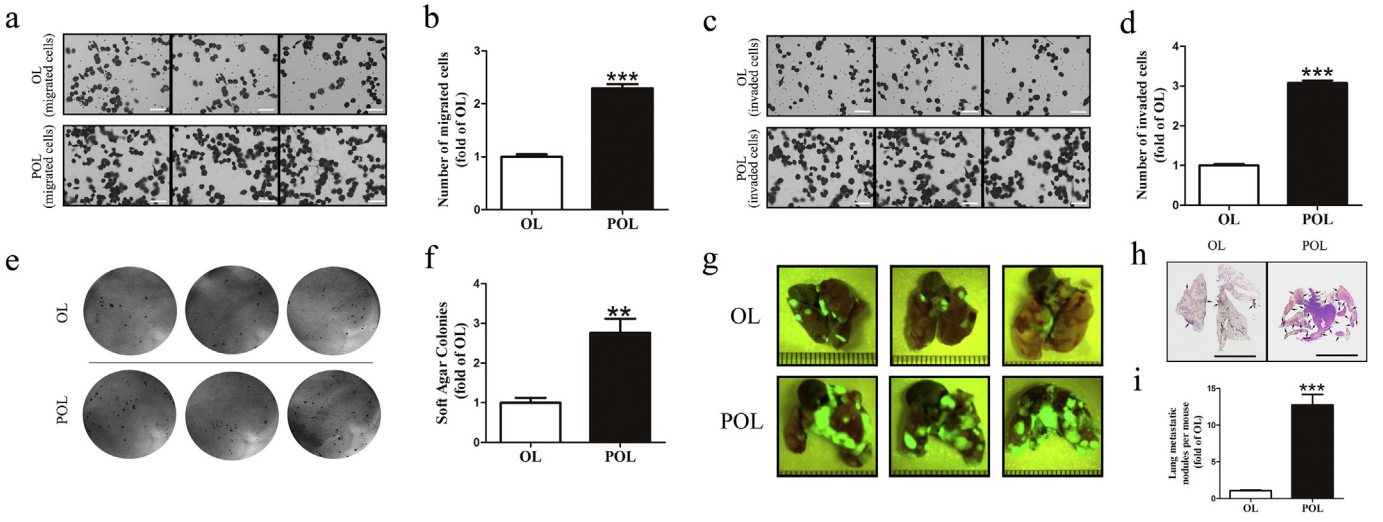

Fig. 1. POL possesses stronger metastatic ability than OL in *in vitro* and *in vivo*. (a) representative images of migrated cells of POL and OL in Transwell migration assay, bar = 60 μm. (b) quantification of transwell migration assay of POL and OL. (c) representative images of invaded cells of POL and OL in Transwell invasion assay with matrigel, bar = 60 μm. (d) quantification of transwell invasion assay with matrigel of POL and OL. (e) representative images of agar colony formation assay of POL and OL. (f) quantification of agar colony formation assay of POL and OL. (g) metastatic nodules in the lungs were shown by whole-lung green fluorescent images, bar = 1 mm. (h) metastatic nodules in the lungs were shown by H&E staining of whole-lung images, arrows show the metastatic nodules, bar = 5 mm. (i) quantification of metastatic nodules in the lungs. (**$P < .01$, ***$P < .001$).

migration and invasion between OL and POL cells were not due to alterations in cell proliferation. MiR-200c expression was detected which showed that the expression of miR-200c in the parental cell line was higher than that in OL and was lower than that in POL (Fig. 2b, $P < .001$). MiR-200c, highly expressed in polymetastatic patients [19] was expressed at higher level in POL cells (Fig. 2b, $P < .001$), showing the clinical relevance of the POL model to clinical polymetastasis. *Sec23a* expression pattern was the opposite of the miR-200c, *i.e.*, it was reduced in POL cells (Fig. 2c-e, $P < .01$). This finding is consistent with the report that *Sec23a* is a confirmed gene target of miR-200c [19,30].

To determine the role of miR-200c and *Sec23a* in regulating the metastatic potential of OL cells, stable miR-200c overexpression (OL-miR-200c-OE cells) and *Sec23a* interference (OL-sh*Sec23a*) were achieved by lentivirus infection (Methods). and altered expression of miR-200c and *Sec23a* was confirmed by PCR (Fig. 3a-b, $P < .001$) and by Western

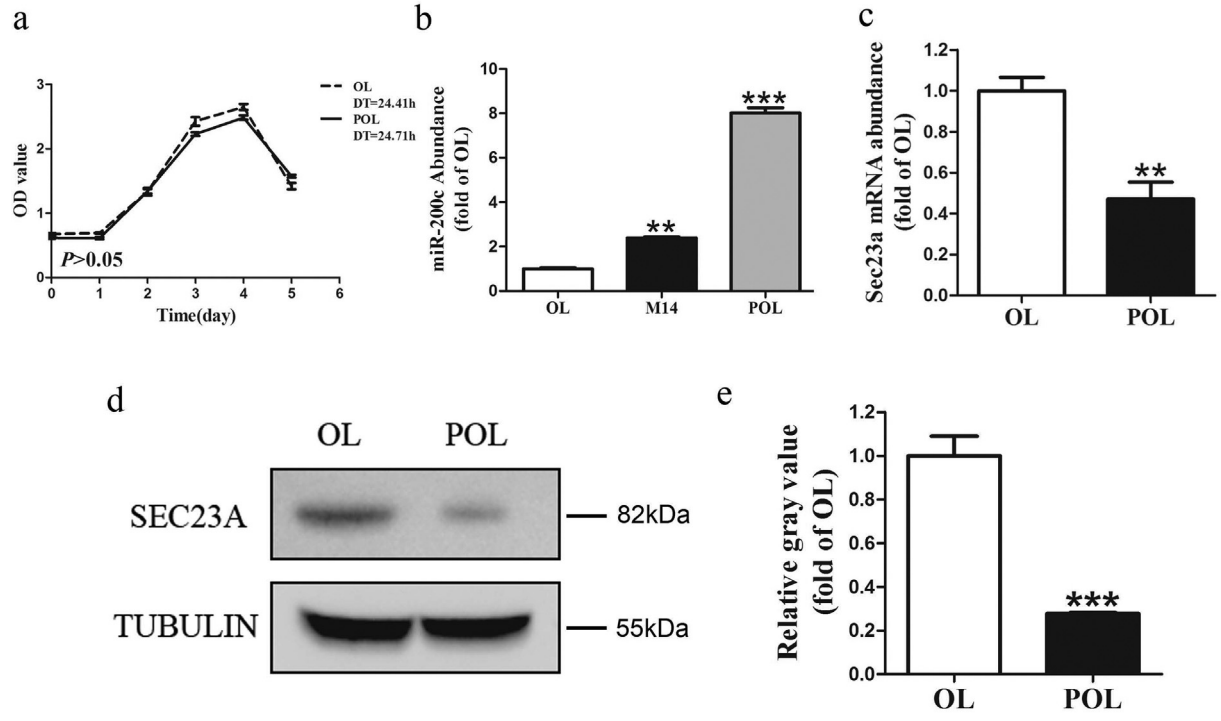

Fig. 2. The expression level of miR-200c in POL is significantly higher than that in OL, and the expression level of *Sec23a* in POL is significantly lower than that in OL. (a) proliferation activities of POL and OL measured by the CCK-8 assay. (b) miR-200c abundance in POL and OL measured by Real-time quantitative PCR. (c) mRNA abundance of *Sec23a* in POL and OL measured by Real-time quantitative PCR. (d) protein abundance of SEC23A in POL and OL measured by Western-blotting. (e) quantitative analysis of the expressions of SEC23A in POL and OL. (**$P < .01$, ***$P < .001$).

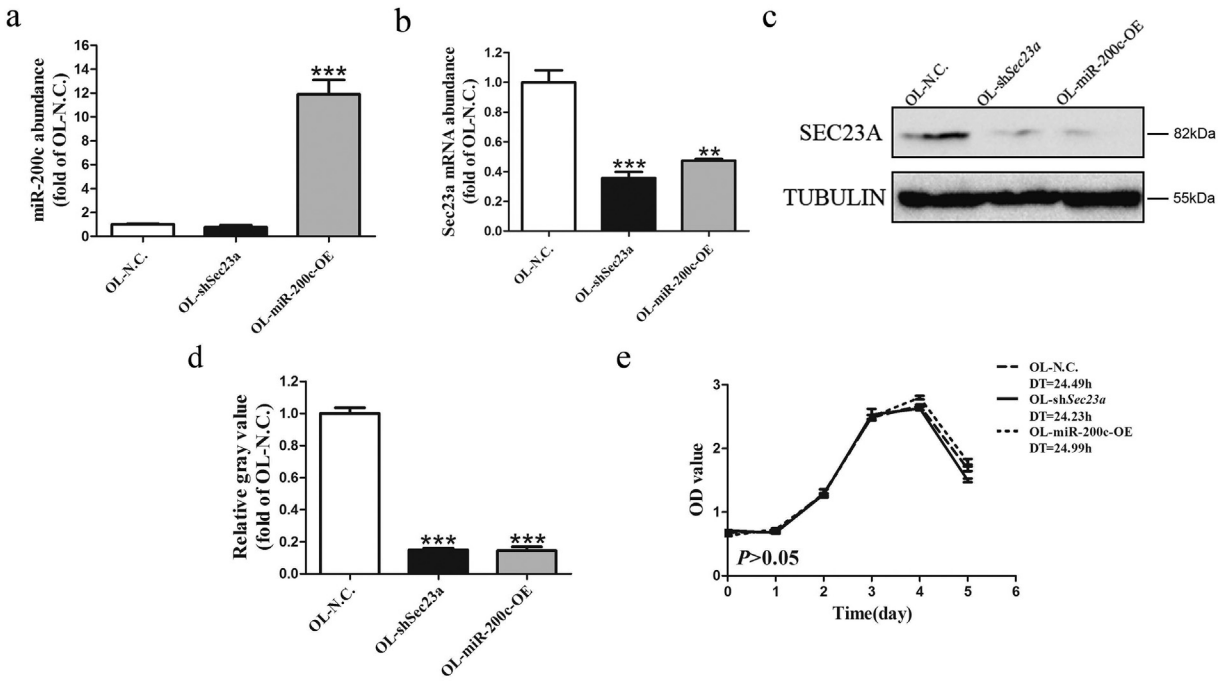

Fig. 3. Overexpression of miR-200c and inhibition of *Sec23a* expression in OL. (a) miR-200c abundance in OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE measured by Real-time quantitative PCR. (b) mRNA abundance of *Sec23a* in OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE measured by Real-time quantitative PCR. (c) protein abundance of SEC23A in OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE measured by Western-blotting. (d) quantitative analysis of the expressions of SEC23A in OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (e) proliferation activities of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE measured by the CCK-8 assay. (**$P < .01$, ***$P < .001$).

blot analysis (Fig. 3c-d, $P < .001$). Altered mIR-200c and *Sec23a* expression had no significant effect on OL cell proliferation (Fig. 3e, $P > .05$).

MiR-200c overexpression and *Sec23a* inhibition lead to comparable level of enhanced cell migration (Fig. 4a-b, $P < .001$), invasion (Fig. 4c-d, $P < .001$) and metastatic colony formation (Fig. 4e-f, $P < .001$) *in vitro*, as well as more pronounced lung metastases *in vivo* (Methods, Fig. 4g-I, $P < .001$). miR-200c overexpression and *Sec23a* inhibition also resulted in the development of more metastatic foci in the thoracic cavities and on the backsides (Supplementary Fig. 2a-b). One mouse injected with the OL-sh*Sec23a* cells even developed a GFP-labeled metastatic foci in one of the testis (Supplementary Fig. 2c). Mice received OL-sh*Sec23a* cells and the OL-miR-200c-OE cells suffered from more severe weight loss (Supplementary Fig. 2d). These observations collectively indicate that overexpression of miR-200c or inhibition of *Sec23a* augmented metastasis both *in vitro* and *in vivo*.

7. MiR-200c over-expression and *Sec23a* interference triggered significant changes in protein secretion

SEC23A is essential for the assembling of COPII, and has also been shown to interact with TRAPP, Grh1p, and Dynactin which are involved in anterograde vesicle transport from ER to Golgi [21,23,31]. Previous studies have reported that *Sec23a* is responsible for the secretion of many extracellular matrix components involved in different physiological processes such as craniofacial development [32,33] and chondrogenesis [34,35]. Since *Sec23a* can be regulated by miR-200c, we hypothesized that over-expression of miR-200c and down-expression of *Sec23a* might profoundly alter the protein secretion function of OL cells, promoting oligometastatic to polymetastatic progression.

Mass spectrometry (MS) detection of secreted proteins with Tandem Mass Tags (TMT) labeling was performed using conditioned media of OL-miR-200c-OE cells, OL-sh*Sec23a* cells, and OL-N.C. cells (Supplementary Fig. 3). 3585 proteins were identified among which 1510 were secreted proteins of which 475 were classified as the classical secreted proteins and the rest 1035 were classified as the nonclassical secreted proteins (Fig. 5a). Heat map and hierarchical clustering indicated that over-expression of miR-200c or down-expression of *Sec23a* dramatically changed the secreted proteome pattern of OL cells (Fig. 5b). Gene Ontology (GO) analysis annotated biological processes related to the functions of the differentially expressed and decreased secreted proteins (Fig. 5c). Protein-protein interactions (PPI) analyses revealed functional relationships within the decreased secreted proteins (Fig. 5d). MS quantitative analysis identified 16 commonly decreased secreted proteins shared by the changed secreted proteomes of OL-sh*Sec23a* cells and OL-miR-200c-OE cells (Fig. 5e-f). To confirm the down-regulation of proteins identified by MS analysis, Thrombospondin, Transferrin, S100A8, CYB5R3, and CXCL4 were selected for the RT-PCR and western-blot validation using the conditioned media, respectively (Fig. 5g-h).

7.1. The effect of miR-200c on metastasis can be reversed by the overexpression of Sec23a

Thus far, we have demonstrated that overexpression of miR-200c and inhibition of *Sec23a* produced comparable metastasis promoting effect as well as similar changes in secretome function in M14 OL cells (Fig. 2-5). Taking in consideration of the knowledge that *Sec23a* is a gene target of miR-200c [19,30], our observations suggest that the mechanism underlying the metastasis promoting effect of miR-200c could be at least in part, mediated by its inhibition of *Sec23a* gene expression. To molecular order *Sec23a* to be functionally downstream of miR-200c, we designed a rescue experiment in which *Sec23a* is overexpressed by lentivirus infection in OL-miR-200c-OE cells that stably overexpress miR-200c (Fig. 6a-c, $P < .001$). Overexpression of *Sec23a* did not alter the proliferation of OL-miR-200c-OE cells (Fig. 6d, $P > .05$). In contrast, overexpression of *Sec23a* effectively and successfully reversed augmented cell migration (Fig. 6e-f, $P < .001$), invasion (Fig. 6g-h, $P < .01$) and colony formation (Fig. 6i-j, $P < .01$) as a result of miR-200 overexpression. More importantly, overexpression of *Sec23a* switched polymetastatic phenotype seen in mice injected OL-miR-200c-OE-NC cells to oligometastasis (Fig. 6k-l, $P < .001$). This set of observations confirm that *Sec23a* functions as a downstream

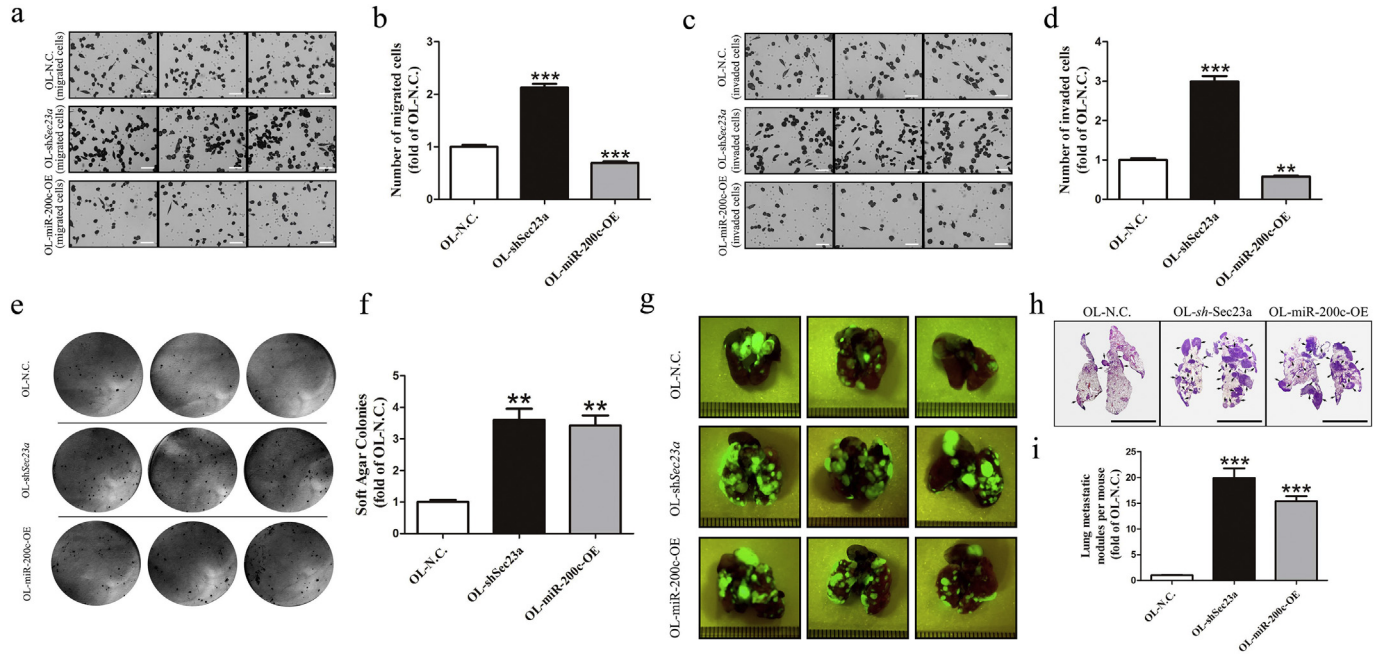

Fig. 4. Overexpression of miR-200c and inhibition of *Sec23a* expression effectively enhance the metastatic ability of OL *in vitro* and *in vivo*. (a) representative images of migrated cells of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE in transwell migration assay, bar = 60 μm. (b) quantification of transwell migration assay of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (c) representative images of invaded cells of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE in transwell invasion assay with matrigel, bar = 60 μm. (d) quantification of transwell invasion assay with matrigel of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (e) representative images of agar colony formation assay of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (f) quantification of agar colony formation assay of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (g) metastatic nodules in the lungs were shown by whole-lung green fluorescent images, bar = 1 mm. (h) metastatic nodules in the lungs were shown by H&E staining of whole-lung images, arrows indicate the metastatic nodules, bar = 5 mm. (i) quantification of metastatic nodules in the lungs. (**$P < .01$, ***$P < .001$).

mediator of miR-200 in the promotion of oligometastatic to polymetastatic progression.

8. Discussion

The introduction of the theory of oligometastatic tumors provides an opportunity to cure metastatic tumors [4,36,37]. Clinical observations show that cases of oligometastatic tumors are more prevalent than expected, for example, the proportion of oligometastatic tumors in non-small cell lung cancer can be as high as 50% [38]. As the sensitivity and accuracy of imaging techniques used in the diagnosis of cancer continue to improve, tumors diagnosed at the oligometastatic phase will continue to increase. Surgery and SBRT have achieved remarkable improvements in the targeted treatments for patients with stable oligometastatic tumors [4,12,39]. However, a large percentage of oligometastatic patients will inevitably progress to polymetastatic phase [40,41]. Clinical outcomes also show that the evolution from oligometastasis to polymetastasis is the root cause of the failure of targeted treatments in oligometastatic tumors [40].

Targeted therapy for oligometastatic tumors has shown great promise for clinical cure of a subset of metastatic tumors. However, the major obstacle that hinders the development of oligometastasis-based targeted therapy is the lack of mechanistic understanding of progression from oligometastasis to polymetastasis. Although elevated miR-200c expression was observed in clinical polymetastases, the context-dependent dual functions (inhibition and promotion) of miR-200c makes it not a feasible therapeutic target for developing oligometastasis-based therapies.

In this study, we characterized the metastasis promoting effect of miR-200c in the context of oligometastatic to polymetastatic progression, providing more experimental evidences for the context-dependency of the role of miR-200c in tumor metastasis. While miR-200c overexpression promotes oligometastatic to polymetastatic progression *in vivo*, it inhibits migration and invasion *in vitro*. The inhibitory effect on migration and invasion *in vitro* is most likely the result of inhibition of epithelial to mesenchymal transition [17,18] as evidenced by changes in cell morphology and surface markers(Supplementary Fig. 5). However, elevated miR-200c promotes metastasis at colonization step in our model, which is consistent with Korpal's findings [19].

Recent studies have shown the participation of secreted proteins in human cancer [42–44]. Secreted proteins can reshape tumor microenvironment, thereby change the metastatic characteristics of the tumor cells at the rate limiting step of metastasis-colonization. The current study, by identifying *Sec23a* as a suppressor of miR-200c augmented olygometastatic to polymetastatic progression, has provided a new theoretical framework for clinical translation. In our previous study, we identified *Sec23a* as a gene target of miR-200c by gene microassay, consistent with Korpal's study in breast cancner [19,27,28]. Korpal et al. first reported the metastasis-promoting activity of miR-200c was reported to be mediated by *Sec23a*'s secretom function [19]. While our findings are highly consistent with that reported by Korpal et al., we characterized the effect of miR-200c/*Sec23*a axis on tumor metastasis in the context of oligometastatic to polymetastatic progression. Further, survival analysis using The Cancer Genome Atlas (TCGA) database showed *Sec23a* as a favorable prognostic marker for skin cutaneous melanoma, supporting the clinical relevance of our findings.

Different secreted proteins identified by MS analysis may be involved in oligometastatic to polymetastatic progression, either as regulators or as biomarkers. For example, THBS1 can affect endothelial cell proliferation, migration, and apoptosis by antagonizing the activity of VEGF, thus inhibit tumor angiogenesis [45,46]. Transferrin behavea as a tumor suppressor in diverse cancers by inhibiting endothelial cell survival [47]. Vitamin D binding Protein GC is negatively correlated with cancer risk [48,49]. The immune chemokines CXCL4 can inhibit tumor

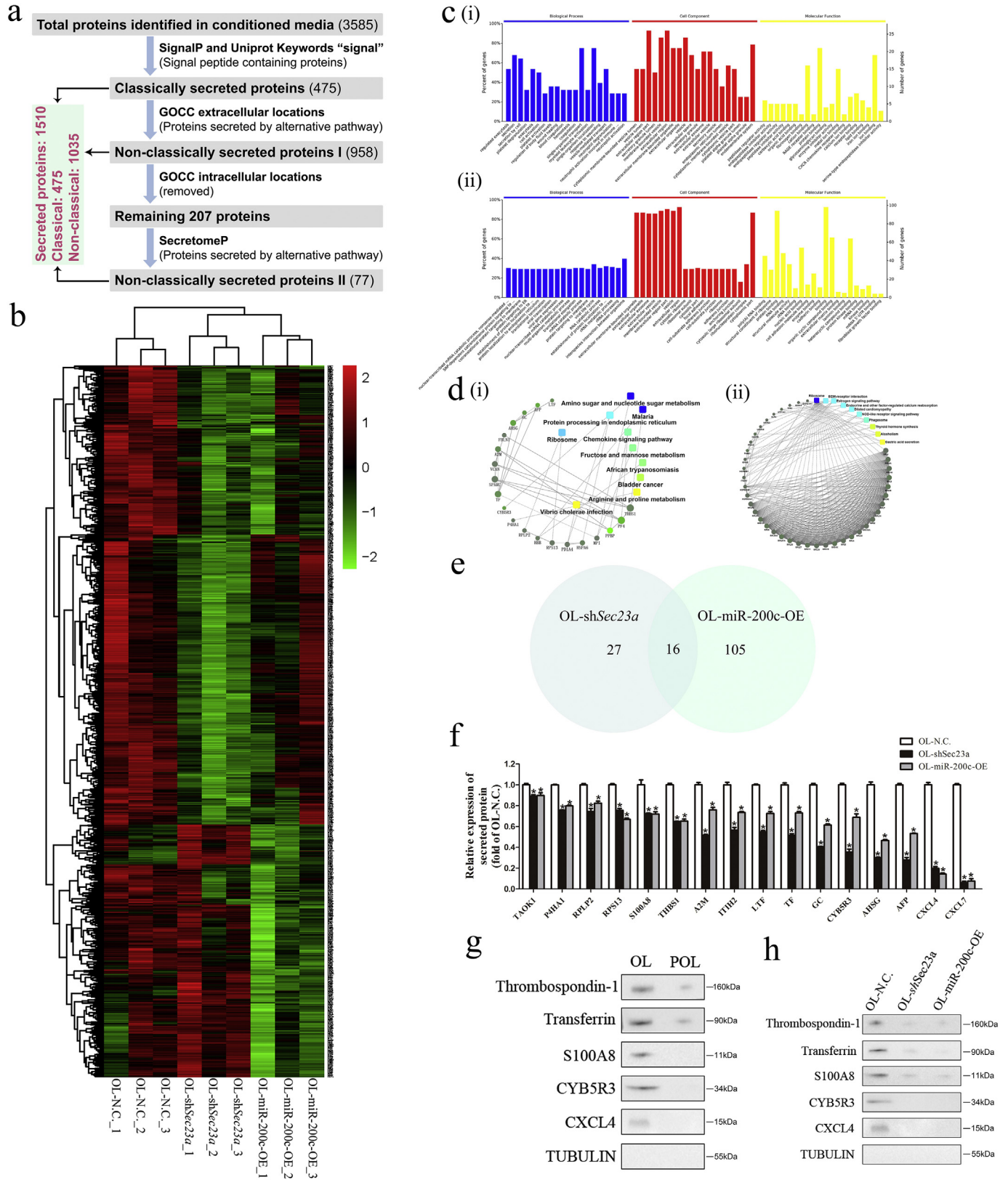

Fig. 5. Mass spectrometry(MS) detection of secreted proteins of OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (a) identification workflow of secretory proteins applied to the MS profiles. (b) heat map and hierarchical clustering of differentially expressed secreted proteins in OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE. (c) GOBP, GOCC and GOMF analysis of the secretory proteomic profiles of OL-sh*Sec23a* *vs* OL-N.C. (i) and OL-miR-200c-OE *vs* OL-N.C.(ii). (d) PPI analysis of the secretory proteomic profiles of OL-sh*Sec23a* *vs* OL-N.C. (i) and OL-miR-200c-OE *vs* OL-N.C.(ii). (e) the number of downregulated secretory proteins of OL-sh*Sec23a* *vs* OL-N.C. and OL-miR-200c-OE *vs* OL-N.C.. (f) quantification of the commonly downregulated secretory proteins of OL-sh*Sec23a* and OL-miR-200c-OE *vs* OL-N.C. (g) validation of the selected down-regulated secretory proteins using the conditioned media from POL and OL by western-blotting. (h) validation of the selected down-regulated secretory proteins using the conditioned media from OL-N.C., OL-sh*Sec23a* and OL-miR-200c-OE by western-blotting. (*$P < .05$).

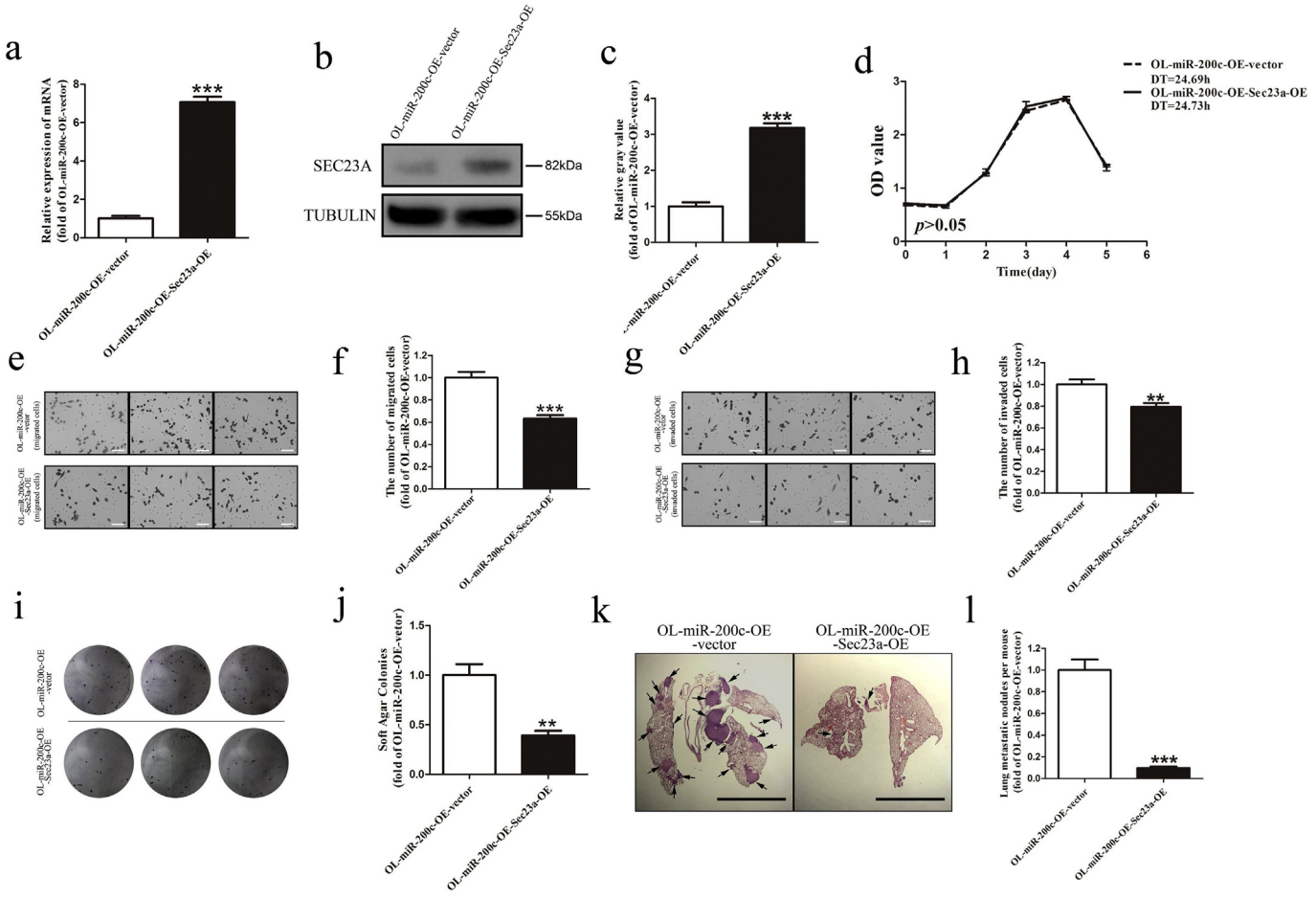

Fig. 6. Overexpression of *Sec23a* in OL-miR-200c-OE recedes its advanced metastatic activity. (a) mRNA abundance of *Sec23a* in OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE measured by Real-time quantitative PCR. (b) protein abundance of SEC23A in OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE measured by Western-blotting. (c) quantitative analysis of the expressions of SEC23A in OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE. (d) proliferation activities of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE measured by the CCK-8 assay. (e) representative images of migrated cells of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE in transwell migration assay, bar = 60 μm. (f) quantification of transwell migration assay of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE. (g) representative images of invaded cells of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE in transwell invasion assay with matrigel, bar = 60 μm. (h) quantification of transwell invasion assay with matrigel of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE. (i) representative images of agar colony formation assay of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE. (j) quantification of agar colony formation assay of OL-miR-200c-OE-vector and OL-miR-200c-OE-Sec23a-OE. (k) metastatic nodules in the lungs were shown by H&E staining of whole-lung images, arrows indicate the metastatic nodules, bar = 5 mm. (l) quantification of metastatic nodules in the lungs. (**$P < .01$, ***$P < .001$).

metastasis by enhancing immune response [50,51]. Therefore, it is reasonable to conclude that reductions of these tumor metastasis suppressors upon *Sec23a* down-regulation by miR-200c overexpression will elicit multi-factorial and multi-functional effects on tumor cell-microenvironment interactions that augment to oligometastatic to polymetastatic progression.

Among the identified secretary proteins regulated by *Sec23a*, S100A8, a regulator of autophagy, may be of particular importance. S100A8 promotes autophagy by competing with BCL-2 binding to BECN1, resulting in the increase of the BECN1-PI3KC3 complex formation and subsequent autophagosome maturation [52]. The role of autophagy in inhibiting tumorigenesis and metastasis has been widely demonstrated, but the underlying mechanisms have not been fully elucidated. Future investigations have been initiated to explore the role of *Sec23a* in metastasis-related autophagic events.

In summary, our work presented here have provided a new theoretical framework for future research to explore the translational potential for using Sec23a as a biomarker for selection of oligometastatic patients for therapies of curative intent. Since many secreted proteins affecting tumor progression can be regulated by Sec23a, whether Sec23a can be a therapeutic target for the development of oligometastasis-based therapies merits further investigation.

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Author contributions

Zhiwei Sun performed the experiments and analyzed data. Zhiwei Sun and Shixia Zhou contributed to the writing of this manuscript. Junlin Tang, Ting Ye, Jingyuan Li and Doudou Liu participated in the conduction of this study. Jian Zhou designed the secretory protein profile analysis. Jianyu Wang and H. Rosie Xing designed this study, oversaw the execution of this study and contributed to the writing and revision of this manuscript.

Conflicts of interest statement

All authors have no conflicting financial interests to declare.

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