Regulation of intercellular biomolecule transfer–driven tumor angiogenesis and responses to anticancer therapies

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Introduction

Within a multicellular organism, the horizontal exchange of biomolecules such as nucleic acids, polypeptides, lipids, and others between cells has emerged as an important mode of communication that encourages collective cell behavior (1). In a healthy organism, biomolecule exchange between diverse types of normal cells helps to maintain homeostatic balance and organize normal biologic processes (e.g., antigen presentation [refs. 2, 3] or normal angiogenesis [ref. 4]). However, in an organism that bears a malignant tumor, this intercellular biomolecule transfer (ICBT) from cancer cells to normal cells often stimulates tumor growth, progression, and metastasis (5–8).

ICBT can occur through a multitude of mechanisms, including uptake of extracellular vesicles and apoptotic bodies, cell fusion, trogocytosis, trans-endocytosis, cell junction and tunneling nanotube formation, and others (reviewed in ref. 9). In the context of tumorigenesis, ICBT mediated by tumor-derived extracellular vesicles (TEVs) has emerged as a pivotal driver of pathogenesis and outcome of oncologic diseases (5, 7). Biomolecules delivered by TEVs reprogram normal cells to contribute to many processes that promote tumor growth and progression, including modulation of metabolic activities, formation of a metastatic niche, suppression of immune responses, stimulation of angiogenesis, etc. (5, 7, 10–12).

Among other types of normal cells, endothelial cells (ECs) can become targets for ICBT. Intratumoral ECs in proximity to malignant cells were found to harbor genetic alterations similar to those found in the malignant cells, and ICBT between these cell types during cell fusion or efferocytosis has been proposed as a putative mechanism underlying this phenomenon (13). Subsequent studies confirmed transfer of tumoral DNA from malignant cells to ECs (14), demonstrated activation of ECs by TEVs (15), and established the paradigm supporting the important role of TEV-mediated ICBT in angiogenesis within tumor microenvironments (reviewed in refs. 5, 8, 16).

Importantly, TEV-mediated ICBT often undermines the efficacy of anticancer therapies (17–19). Furthermore, radio- and chemotherapeutic agents increase production and/or release of TEVs by malignant cells; this increase is implicated in the iatrogenic metastatic disease arising from the treatment of primary tumors (20–25). Addressing these challenges requires a better understanding of genetic regulators of mechanisms underlying the protumorigenic ICBT. In addition, development of pharmacologic means to restrict ICBT should offer a novel approach to curtail tumor growth and progression and to improve the efficacy of existing anticancer therapies.

The biological barriers against ICBT are expected to preserve functional integrity of normal cells and restrict their cooperation...
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with malignant cells. The type I interferon (IFN) pathway acts as one of those barriers that prevents generation of the prometastatic niche and pulmonary metastases (26–28). Cholesterol 25-hydroxylase (CH25H), an enzyme that is induced by IFN (29–31), acts to catalyze the formation of 25-hydroxycholesterol (25HC). This oxysterol inhibits lipid membrane fusion (32) and, accordingly, uptake of TEVs (26). Uptake of TEVs by normal cells was also shown to be inhibited by the antihypertensive agent reserpine, which in turn could restore CH25H expression otherwise downregulated by TEVs (26).

We next examined ICBT in vitro mediated by isolated TEVs characterized in Supplemental Figure 1, E–G. To safeguard against possible peculiarities of the intratumoral ECs in the GFP-expressing mice, we isolated ECs from the lungs of naïve mice (Supplemental Figure 1, H and I) and treated them with TEVs isolated from B16F10 cells stably expressing GFP. A greater amount of Gfp mRNA was transferred into CH25H-deficient ECs compared with WT ECs; however, this phenotype was partially reversed upon treatment with reserpine (Figure 1E). Similar results were obtained when ICBT was assessed by transfer of DiD dye from TEVs into ECs (Supplemental Figure 1, J and K). Collectively, these results suggest that CH25H acts as a genetic suppressor of ICBT between malignant cells and ECs and characterize reserpine as a pharmacologic agent capable of inhibiting ICBT in vitro and in vivo.

Inactivation of stromal CH25H promotes tumor growth and angiogenesis. CH25H levels have been found to be decreased in leukocytes from tumor-bearing mice and melanoma patients compared with tumor-free control groups (26); however, the importance of CH25H downregulation in normal cells for tumor growth is not completely understood. Intriguingly, during the course of experiments described in Figure 1A, we noticed that subcutaneous TdTomato-expressing B16F10 melanoma tumors grew faster in Ch25h–/– compared with WT mice (Figure 2, A and B). Similar results were obtained with tumors formed by parental B16F10 cells (Supplemental Figure 2, A and B). This observation prompted us to examine the importance of CH25H expression in the tumor microenvironment for growth of other types of cancer cells.

An accelerated growth of syngeneic tumors in Ch25h–/– (compared with WT) mice was observed for transplanted M16499c4 pancreatic ductal adenocarcinoma (Figure 2C) and MC38 colon adenocarcinoma (Figure 2D and Supplemental Figure 2C). Furthermore, this phenotype was not limited to subcutaneous tumors, as Ch25h–/– (compared with WT) mice exhibited a significantly faster growth of orthotopically transplanted MC38 colon tumors (Figure 2E) and TRAMP-C2-luciferase prostate neuroendocrine tumors (Figure 2F and Supplemental Figure 2D). Collectively, these data indicate that stromal CH25H inhibits growth of different types of solid tumors.

Visual appearance of tumors growing in Ch25h–/– mice was suggestive of a greater extent of vascularization (as compared with WT; Figure 3A and Supplemental Figure 3A). This phenotype, as well as an increased ICBT involving ECs in Ch25h–/– mice (Figure 4), prompted us to examine the status of CH25H and angiogenesis in the stromal compartment of human colorectal cancers (CRCs).

Analysis of tumors from an initial cohort of CRC patients (Cohort 1) revealed that levels of CH25H were significantly downregulated in the stromal compartment of malignant colorectal tumors compared with stroma of benign adjacent colon tissue (Figure 3, B and C). Importantly, survival analysis in a cohort of CRC patients with available clinical follow-up data (Cohort 2) revealed that low levels of CH25H within the tumor stroma were significantly associated with highly unfavorable prognosis (HR, 4.3; 95% CI, 1.58–11.57; P = 0.004; Figure 3D). Moreover, containing of CH25H and CD31 revealed that CH25H levels specifically in the CD31+ ECs were notably downregulated in ECs within tumor stroma compared with ECs from healthy colon stroma, as shown first in an analysis of unmatched cases (Cohort 3, Figure 3E). These findings were further validated in an independent set of CRC cases with matched cancer stroma and nearby normal colon stroma (Cohort 4, Figure 3E). These results provide strong correlative support for the notion that downregulation of CH25H occurs in human CRC stroma and, particularly, in the intratumoral ECs, and that this inactivation promotes tumor progression in human CRCs.

Results

CH25H and reserpine inhibit ICBT between malignant cells and ECs. We previously reported that uptake of DiD dye–labeled TEVs is decreased in normal, wild-type (WT) splenocytes pretreated with reserpine or in splenocytes from knockin mice expressing a stabilized mutant of IFN receptor 1 (IFNARI) (33). Importantly, the latter phenotype was lost upon ablation of Ch25h (26). Given that, in addition to TEVs, there are other mechanisms of biomolecule exchange such as uptake of apoptotic bodies, cell fusion, trans-endocytosis, etc. (9), we sought to determine the role of CH25H expression in benign cells in regulating ICBT within solid tumors in vivo.

To this end, we grew subcutaneous tumors from B16F10-TdTomato melanoma cells in the flanks of WT or Ch25h–/– mice that constitutively expressed green fluorescent protein (GFP) (Figure 1A). Tumors were harvested, dissociated, and analyzed for the numbers of TdTomato+GFP+ double-positive cells among immune CD45+ cells and CD45– nonimmune populations (Supplemental Figure 1A). We found that tumors from the Ch25h–/– mice contained a greater number of TdTomato+GFP+ cells in the CD45– nonimmune stromal population (Figure 1B). Subsequent analysis of double-positive cells specifically in fibroblastic (PDGFRe+; Supplemental Figure 1C) and endothelial (CD31+; Supplemental Figure 1D) compartments revealed that ECs are the main target for the CH25H-regulated exchange of biomolecules with malignant cells (Figure 1C). The extent of ICBT in the ECs from B16F10-TdTomato tumors growing in GFP+ mice was notably decreased by in vivo administration of reserpine (Figure 1D).

We next examined ICBT in vitro by treating isolated TEVs characterized in Supplemental Figure 1, E–G. To safeguard against possible peculiarities of the intratumoral ECs in the GFP-expressing mice, we isolated ECs from the lungs of naïve mice (Supplemental Figure 1, H and I) and treated them with TEVs isolated from B16F10 cells stably expressing GFP. A greater amount of Gfp mRNA was transferred into CH25H-deficient ECs compared with WT ECs; however, this phenotype...
To validate these data from human patients in mouse models we examined the role of CH25H in development of the intratumoral vasculature. A greater number of CD31+ ECs was found in B16F10 melanoma tumors grown in Ch25h–/– compared with WT mice (Figure 3, F and G). Furthermore, ablation of CH25H in the tumor microenvironment resulted in a greater number and increased length of blood vessels within these tumors (Figure 3G). Similar results were obtained in subcutaneous tumors of equal size formed by pancreatic adenocarcinoma cells (Supplemental Figure 3B) or MC38 colon adenocarcinoma cells (Supplemental Figure 3C), as well as in MC38 tumors transplanted orthotopically (Supplemental Figure 3D). Analysis of functionality of these blood vessels by injection of FITC-lectin revealed that knockout of CH25H increased perfusion in these tumors (Figure 3H). In all, these results suggest that inactivation of CH25H in the tumor microenvironment stimulates angiogenesis and increases tumor vascularization.

A limited screen for genes known to control tumor angiogenesis revealed a comparable level of Vegfa, Vegfr2, Tie2, Glut1, Mmp9, and Fgf1 mRNA in B16F10 tumors grown in WT and Ch25h–/– mice (Figure 3I). Intriguingly, we detected a relatively greater expression of angiopoietin-2 (Angpt2) in tumors from Ch25h–/– mice compared with those from WT animals. Angpt2 was also increased in MC38 tumors from Ch25h–/– mice (Figure 3I), further suggesting that inactivation of stromal CH25H stimulates the expression of Angpt2 in solid tumors.

Importantly, an increase in Angpt2 induced by TEVs and reversed by reserpine was also independently detected in an RNA sequencing–based profiling of gene expression in CH25H-deficient primary mouse lung ECs (Figure 4A). In addition to Angpt2, 812 out of 2998 differentially expressed genes were induced by TEVs unless pretreated with reserpine (10 μM) followed by a 12-hour exposure to TEVs (20 μg/mL) isolated from GFP+B16F10 cells (n = 4 for each group). Data are presented as mean ± SEM. Statistical analysis was performed using 1-way ANOVA with Tukey’s multiple-comparison test (B, D, and E) or 2-tailed Student’s t test (C). NS, not significant. Experiments were performed independently at least 3 times.
and Ch25h–/– ECs treated with vehicle, tumor cell–conditioned media lacking TEVs, or with recombinant VEGF (Figure 4F and Supplemental Figure 4C). These results suggest that CH25H does not indiscriminately suppress activation of ECs but specifically limits their activation by ICBT.

Indeed, treatment with purified TEVs triggered a more robust in vitro activation of CH25H-deficient ECs compared with their WT counterparts. These phenotypes included an increased TEV-induced proliferation manifested by either cell numbers (Figure 4G) or percentage of Ki67-positive cells (Supplemental Figure 4D) and a greater migration (Supplemental Figure 4E) in Ch25h–/– ECs. Furthermore, compared with WT, Ch25h–/– ECs were more adept in forming endothelial tubes upon treatment with TEVs from MC38 (Figure 4H) or B16F10 (Supplemental Figure 4F) cancer cells. Importantly, neutralization of ANGPT2 prevented in Ch25h–/– ECs (Figure 4C). Importantly, pretreatment with reserpine reversed the TEV-induced increase in Angpt2 mRNA (Figure 4D) and protein (Figure 4E) in Ch25h–/– ECs. These results link ICBT in ECs with induction of ANGPT2.

The angiostatic and antitumorigenic roles of endothelial CH25H. ANGPT2 is produced by intratumoral ECs to facilitate angiogenesis (reviewed in refs. 34–36). Given that robust induction of Angpt2 in tumors from CH25H-deficient mice in vivo (Figure 3I) can be faithfully recapitulated in the cultures of primary mouse lung ECs (Figure 4, D and E), we next used this in vitro system to further interrogate the importance of CH25H in regulating angiogenic activity. Treatment with MC38 tumor cell–conditioned media elicited a greater increase in tube formation by the Ch25h–/– ECs, as compared with WT cells (Figure 4F and Supplemental Figure 4C). Importantly, we detected no difference in activities of WT and Ch25h–/– ECs treated with vehicle, tumor cell–conditioned media lacking TEVs, or with recombinant VEGF (Figure 4F and Supplemental Figure 4C). These results suggest that CH25H does not indiscriminately suppress activation of ECs but specifically limits their activation by ICBT.
Figure 3. The angiostatic role of CH25H in the tumor microenvironment. (A) A representative image of B16F10-TdTomato tumors and surrounding blood vessels in GFP-WT and GFP-Ch25h−/− mice. (B) Representative image of blood vessels (green) and CH25H (red) in normal stroma and colorectal cancer stroma. Scale bar: 100 μm. (C) Scatterplot of quantitative stromal CH25H protein expression levels in normal adjacent stroma and tumor stroma (Cohort 1). (D) Kaplan-Meier survival analysis of CRC stromal CH25H protein levels, dichotomized into high and low CH25H expression, indicating increased risk of recurrence with loss of CH25H protein levels (Cohort 2). (E) Scatterplot of quantitative CH25H protein levels within the endothelium of normal adjacent stroma and CRC stroma (Cohort 3, left panel) and the validation of endothelial CH25H expression levels between paired samples of normal adjacent stroma and CRC stroma (Cohort 4, right panel). (F) Analysis of CD31+ ECs in B16F10 tumors (s,c., 1 × 10^6 cells/mouse) of comparable volume grown for approximately 2 weeks in WT (n = 4) and Ch25h−/− (n = 5) mice. Scale bar: 100 μm. (G) Quantification of data from experiment described in panel F. Data averaged from 5 random fields in sections from each of 4 or 5 animals are shown. (H) Representative Image (left) of localization of blood vessels (red) and lectin+ (green) area in tumor from WT and Ch25h−/− mice after injection with FITC-lectin (i.v., 100 μg/mouse). Quantification (right) of FITC-positive area (n = 5 for each group) of the images. Scale bar: 50 μm. (I) qPCR analysis of relative expression indicated genes in B16F10 (n = 5) and MC38 (n = 6) tumor tissues from WT and Ch25h−/− mice. For each gene, mRNA levels in WT tumors were defined as 1.0. Data are presented as mean ± SEM. Statistical significance was determined by 2-tailed Student’s t test (C, E, and G–I) or log-rank (Mantel-Cox) test (D). Experiments were performed independently at least 3 times.
an increase in tube formation in response to tumor-conditioned media (Figure 4F) or purified TEVs (Figure 4H), indicating that ANGPT2 is required for the ICBT-induced activation of ECs. Furthermore, ICBT-driven hyperactivation of Ch25h–/– ECs could be effectively reversed by either reexpressing CH25H or by treating these cells with the end-product of the CH25H enzymatic activity — 25HC (Figure 4I). Collectively, these results suggest that CH25H acts as a biological barrier that restricts the ICBT-induced ANGPT2-dependent activation of ECs.

These results, together with the protumorigenic phenotypes observed in Ch25h–/– mice (Figure 2), prompted us to concentrate on specific roles of endothelial CH25H in vivo. To this end, we iso-
lated ECs from the lungs of naïve GFP+ WT or Ch25h–/– mice and coinjected these cells with B16F10 cells into the flanks of WT mice. Under these conditions, at least some of the transferred GFP+ ECs were incorporated into intratumoral blood vessels (Figure 5A). Whereas ECs of both genotypes accelerated tumor growth, Ch25h–/– ECs were significantly more active in these settings (Figure 5B and Supplemental Figure 5A), indicating that endothelial CH25H interferes with the ability of ECs to support tumor growth.

To further test this hypothesis, we generated a conditional knockout allele of Ch25h by flanking the sole exon of this gene with loxP sites (Supplemental Figure 5B). We crossed these mice with animals that express Cre recombinase under the EC-specific VE-cadherin promoter (37); the resulting animals lacked CH25H in primary lung ECs but not fibroblasts (Supplemental Figure 5C). Whereas no obvious vascular alterations or other abnormalities were detected in these naive animals, they displayed a notable phenotype when challenged with subcutaneous tumors. Under these conditions, efficient ablation of CH25H was achieved in the intratumoral ECs (Supplemental Figure 5D). Importantly, growth of B16F10 melanoma tumors was significantly accelerated in mice lacking CH25H in the ECs (Figure 5, C and D). Furthermore, ablation of CH25H in ECs notably increased intratumoral angiogenesis, as evident from an increased CD31+ area as well as a greater number of blood vessels and an increase in their length (Figure 5, E and F). Similar observations were made in experiments involving MC38 colon adenocarcinoma tumors (Supplemental Figure 5, E–H). In all, these results suggest that endothelial CH25H plays important angiostatic and antitumorigenic roles.

Reserpine inhibits tumor angiogenesis and improves the outcome of radio- and chemotherapies. We have previously reported that treatment with reserpine increases the expression of CH25H in TEV-treated cells (26). Thus, we utilized this agent as a complementary pharmacologic tool to ascertain the importance of ICBT-driven endothelial activation and tumor angiogenesis.
Used at previously described low doses (26), reserpine was well tolerated and did not cause sleepiness or decrease animal weight while inhibiting the intratumoral ICBT (Figure 1, D and E). Furthermore, in vitro pretreatment of ECs with reserpine suppressed TEV-induced expression of Angpt2 (Figure 4, B, D, and E) and significantly inhibited endothelial tube formation (Supplemental Figure 6A). These results prompted us to examine the effect of reserpine on tumor angiogenesis in vivo.

Administration of reserpine did not decrease expression of Angpt1 or Tie2 in the B16F10 tumors growing in WT or Angpt1 or reserpine on tumor angiogenesis in vivo. (Figure 6A). These results prompted us to examine the effect of reserpine on tumor angiogenesis in vivo.

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between malignant cells and CH25H-deficient ECs (Figure 1) might be reflective of ECs representing the first barrier encountered by the circulating tumor-derived matter. CH25H is induced by IFN, and downregulation of its receptor often occurs in many types of cancer (28, 49–51) and contributes to angiogenic activation by VEGF (52). Importantly, the loss of CH25H per se does not

Discussion

Endothelial CH25H as a genetic suppressor of ICBT-driven intratumoral angiogenesis. Given the paramount role of ICBT in tumor growth, progression, and resistance to therapeutics (5–7), it is of critical importance to understand how ICBT is regulated and could be pharmacologically controlled in vivo. An increased ICBT

Figure 6. Angiostatic and antitumorigenic effects of reserpine in solid tumors. (A) qPCR analysis of relative Angpt2 mRNA levels in B16F10 tumors from WT and Ch25h−/− mice treated with vehicle or reserpine (1 mg/kg, i.p. every other day for 4 days). n = 5 for each group. (B) Representative immunofluorescence images and quantification of CD31-positive areas in B16F10 tumors from WT and Ch25h−/− mice (n = 5 for each group) treated with vehicle or reserpine as described in panel A. Scale bar: 100 μm. (C) Growth of human HCT116 tumors (inoculated s.c. at 5 × 10⁶ cells/mouse) in NSG mice treated with vehicle or reserpine (1 mg/kg) every other day. n = 5 for each group. (D) Representative immunofluorescence image (upper) of CD31 staining of HCT116 tumors from NSG mice treated with vehicle or reserpine. Quantification (bottom) of CD31-positive areas and average distance of blood vessels. Quantification averaged from 5 random fields in sections from each of 5 animals is shown (n = 5 for each group). Scale bar: 100 μm. (E) Analysis of B16F10 tumor growth (inoculated s.c. at 1 × 10⁶ cells/mouse) in WT and Ch25h−/− mice (n = 5 for each group) followed by vehicle or reserpine treatment (1 mg/kg, i.p. every other day). (F) Analysis of B16F10 tumor mass on day 15 after inoculation (s.c. at 1 × 10⁶ cells/mouse) into indicated mice (n = 5 for each group) followed by vehicle or reserpine treatment (1 mg/kg, i.p. every other day). Data are presented as mean ± SEM. Statistical analysis was performed using 1-way ANOVA with Tukey’s multiple-comparison test (A, B, F, and G), 2-way ANOVA with Tukey’s multiple-comparison test (C and E), or 2-tailed Student’s t test (D). NS, not significant. Experiments were performed independently at least 3 times.
redundantly responsible for increased ANGPT2 and resulting angiogenesis in the absence of endothelial CH25H. Under these conditions, induction of ANGPT2 upon inactivation of CH25H is likely triggered by an increased extent of ICBT as well as by additional CH25H-dependent mechanisms that affect TEV-sensitive and/or reserpine-sensitive transcription factors such as SP1, EGR1, ELF1, and GATA2 and related pathways uncovered in our study (Figure 4, A and B). Intriguingly, cyclosporin A, an inhibitor of the calcineurin/NFAT /ANGPT2 signaling axis (63), also had a modest effect on ANGPT2 induction by TEVs (Figure 4D), suggesting that diverse and likely redundant pathways mediate the effects of ICBT.

It is likely that the importance of endothelial CH25H for suppressing tumor angiogenesis and growth gleaned from experiments using knockout models is seriously underestimated because of notable decreases in CH25H levels in the intratumoral ECs, as detected in human CRC patients (Figure 3, B–D). This downregulation of CH25H in the tumor microenvironment was significantly associated with poor prognosis (Figure 3D) and could be mediated by downregulation of CH25H expression in response to TEVs (26) as well as by additional, yet to be delineated mechanisms.

CH25H catalyzes monooxygenation of cholesterol into 25HC. Its absence may promote formation of other types of oxycholesterols such as proangiogenic 27-hydroxycholesterol (53). Nevertheless, evidence demonstrating the ability of 25HC to directly suppress tube formation (Figure 4I) and EC proliferation (54) suggests a key role for 25HC in the angiostatic phenotype. It is important to note that 25HC inhibits lipid membrane fusion (32), which is essential for cell fusion, uptake of TEVs and apoptotic bodies, cell junction and tunneling nanotube formation, and other events enabling ICBT (reviewed in ref. 9). Whereas the importance of CH25H and 25HC in restricting the uptake of TEVs has been demonstrated (26), future studies will focus on determining specific roles of these regulators in other mechanisms of ICBT.

Numerous mediators of angiogenesis delivered to ECs by extracellular vesicles from either normal or malignant cells include VEGF-A and -D, WNT4, IL-8, carbonic anhydrase 9, diverse types of noncoding RNAs, and others (55–62). It is likely that more than one type of these diverse biomolecules is redundantly responsible for increased ANGPT2 and resulting angiogenesis in the absence of endothelial CH25H. Under these conditions, induction of ANGPT2 upon inactivation of CH25H is likely triggered by an increased extent of ICBT as well as by additional CH25H-dependent mechanisms that affect TEV-sensitive and/or reserpine-sensitive transcription factors such as SP1, EGR1, ELF1, and GATA2 and related pathways uncovered in our study (Figure 4, A and B). Intriguingly, cyclosporin A, an inhibitor of the calcineurin/NFAT /ANGPT2 signaling axis (63), also had a modest effect on ANGPT2 induction by TEVs (Figure 4D), suggesting that diverse and likely redundant pathways mediate the effects of ICBT.

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Reserpine as a pharmacologic inhibitor of ICBT and, potentially, a component of anticancer therapies. Importantly, the ICBT-driven ANGPT2 expression, EC activation, and intratumoral angiogene-
Analysis of plasma membrane polarization in WT and Ch25h–/– μC)

MC38 cancer cells treated with vehicle or reserpine (10 μM for 12 hours). (A) qPCR analysis of Rab7, Rab11b, Rab27a, Sdc18p, Arf6, Ykt6, Snap23, Hgs, and Pdcd6ip relative levels (n = 4 for each group) in MC38 cancer cells treated with vehicle or reserpine (10 μM for 12 hours). (B) Analysis of plasma membrane polarization in WT and Ch25h–/– ECs treated with reserpine (10 μM) for 12 hours. (C) Flow cytometric analysis of percentage of DII-CD31+ cells upon incubation of indicated ECs with DII-labeled liposomes (1 μg/mL) in the presence or absence of reserpine (10 μM) for 8 hours (n = 5 for each group). Data are presented as mean ± SEM. Statistical analysis was performed using 1-way ANOVA with Tukey’s multiple-comparison test (A, C, and D) or 2-tailed Student’s t test (B). NS, not significant. Experiments were performed independently at least 3 times.

Figure 8. Mechanism of reserpine-mediated TEV uptake inhibition. (A) Quantification of numbers (upper panel) and total protein content (bottom panel) of TEVs released by the indicated cells following treatment with vehicle or reserpine (10 μM for 48 hours) in vitro. (B) qPCR analysis of Rab7, Rab11b, Rab27a, Sdc18p, Arf6, Ykt6, Snap23, Hgs, and Pdcd6ip relative levels (n = 4 for each group) in MC38 cancer cells treated with vehicle or reserpine (10 μM for 12 hours). (C) Flow cytometric analysis of percentage of DII-CD31+ cells upon incubation of indicated ECs with DII-labeled liposomes (1 μg/mL) in the presence or absence of reserpine (10 μM) for 8 hours (n = 5 for each group). Data are presented as mean ± SEM. Statistical analysis was performed using 1-way ANOVA with Tukey’s multiple-comparison test (A, C, and D) or 2-tailed Student’s t test (B). NS, not significant. Experiments were performed independently at least 3 times.

Perhaps even more exciting is the potential for clinical use of reserpine to improve the efficacy of other types of anticancer treatment, including chemo- and radiotherapies supported by preclinical data presented here (Figure 9). Reserpine has been widely used as a drug for treatment of hypertension and has also been chosen for its ability to inhibit vesicular monoamine transporter-2 and vesicular reuptake (42). We have previously reported that treatment with reserpine increases the expression of CH25H in TEV-treated cells (26). Here we show that reserpine limits the ICBT between malignant and benign cells in vivo (Figure 1) by likely more than one mechanism, including suppression of TEV uptake (26) and a decrease in circulating TEVs (Figure 9A and Supplemental Figure 9A), which can be plausibly attributed to altered TEV production/loading in vitro (Figure 8A). Molecular mechanisms underlying the inhibition of ICBT by reserpine are likely to involve effects on expression of genes involved in production of extracellular vesicles and on plasma membrane fluidity and its ability to fuse with lipid membrane vesicles (Figure 8, B–D). Potential effects of reserpine on additional modes of ICBT (e.g., cell fusion or formation of tunneling nanotubes) require further studies.

Although reserpine elicited only a modest effect on proliferation of malignant cells in vitro (26), additional ICBT-independent mechanisms by which this agent may help to suppress angiogenesis and growth of solid tumors cannot be excluded. Regardless of these specific mechanisms, it is important to note that reserpine robustly suppressed the increase in metastatic disease triggered by therapeutic regimens mostly designed to efficiently eradicate primary tumors (Figure 9).

The relatively low cost of reserpine therapy adds to its benefits, which could be especially important to battle metastatic disease in economically disadvantaged patients worldwide. Given that reserpine is well tolerated and has already been approved for use in human patients, this drug can be immediately tested in clinical trials for inclusion in standard regimens used in the treatment of solid tumors. Furthermore, future development of novel means to control ICBT and improve the outcome of anticancer therapies hold additional promise.

Methods

Detailed methods can be found in the supplemental material.

Human CRC specimens and their analyses. Human CRC tissue microarrays, consisting of formalin-fixed, paraffin-embedded tissue cores, were stained for CH25H. The detailed description of Cohorts 1–4 is provided in the supplemental material. CH25H, CD31, and cytokeratin detection was performed using immunofluorescence and immunohistochemistry, as previously described (64). Quantitative biomarker analysis was performed using Tissue Studio image analysis software (Definiens) to identify epithelial or stromal regions, facilitated by DAPI-stained cell nuclei and cytokeratin-stained cancer cells.

Animal studies. Besides NSG, all other mouse strains (including WT, Ch25h–/–, TgN(AcbtEGFP)1Osb/f), and VE-cadherin–Cre (65) purchased from The Jackson Laboratory were on the C57BL/6J background. The conditional Ch25h allele was created by flanking the single exon of the Ch25h gene with loxP sites inserted into the noncon-
were gifted. All cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and L-glutamine.

TEV isolation, characterization, and assessment of uptake and production were carried out as previously described (10, 26). Briefly, TEVs were collected from (extracellular vesicle–free) media (Gibco, 11965-084) supernatants by ultracentrifugation and additional purification was carried out using discontinuous iodixanol gradients. TEVs were spun over 10%/20%/30% iodixanol layers at 350,000 g (52,000 rpm with Beckman SW 55 Ti rotor), 4°C, for 120 minutes. Then, 10 fractions of 260 μL each were collected starting from the top of the tube, diluted with 1 mL PBS, and resedimented at 100,000 g and 4°C for 70 minutes (Beckman Optima Ultracentrifuge and TLA-100.2 rotor at 53,000 rpm). Pellets in each fraction were resuspended in PBS.

Cell culture. Human 293T and HCT116 cells and mouse B16F10 cells were purchased from ATCC. Mouse MC38 colon adenocarcinoma (from S. Ostrand-Rosenberg, University of Maryland, Baltimore, Maryland, USA), TRAMP-C2-luc prostate neuroendocrine tumor cells (from L. Languino, Thomas Jefferson University, Philadelphia, Pennsylvania, USA), and MH6499c4 pancreatic ductal adenocarcinoma (from B. Stanger, University of Pennsylvania, Philadelphia, Pennsylvania, USA) were gifted. All cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and L-glutamine.

TEV isolation, characterization, and assessment of uptake and production were carried out as previously described (10, 26). Briefly, TEVs were collected from (extracellular vesicle–free) media (Gibco, 11965-084) supernatants by ultracentrifugation and additional purification was carried out using discontinuous iodixanol gradients. TEVs were spun over 10%/20%/30% iodixanol layers at 350,000 g (52,000 rpm with Beckman SW 55 Ti rotor), 4°C, for 120 minutes. Then, 10 fractions of 260 μL each were collected starting from the top of the tube, diluted with 1 mL PBS, and resedimented at 100,000 g and 4°C for 70 minutes (Beckman Optima Ultracentrifuge and TLA-100.2 rotor at 53,000 rpm). Pellets in each fraction were resuspended in PBS.

Figure 9. Administration of reserpine improves the outcomes of radio-/chemotherapies. (A) ELISA analysis of CD63 levels in the plasma from mice bearing B16F10 tumors of similar volume exposed or not to ionizing radiation (IR, 12 Gy), reserpine (1 mg/kg), or both. n = 4 for all groups. (B) Analysis of tumor volume in B16F10 tumors (inoculated s.c. at 1 × 10⁵ cells/mouse) in WT mice treated with vehicle or reserpine (1 mg/kg) upon reaching 30 to 50 mm³. Three days later, vehicle- and reserpine-treated mice with similar tumor volumes underwent 12-Gy irradiation and continued their assigned vehicle or reserpine treatments 3 times per week (n = 4). (C) Kaplan-Meier survival analysis of B16F10 tumor–bearing mice treated as described in panel B until tumors reached 2000 mm³ (n = 4). (D) Quantification of total area of B16F10 metastatic load in lungs from mice described in panel B (n = 4). (E) Schematic of the experiments combining reserpine with FOLFOX treatment of orthotopically inoculated MC38 colon tumors. (F) Representative images and the mass of MC38 tumors from animals treated as in panel E. n = 5 for each group. (G) Representative images of livers from MC38 tumor–bearing mice described in panel E. Arrowheads show macroscopic metastatic lesions found in 8% of vehicle-treated animals, 57% of FOLFOX only–treated animals, and none of the animals that received reserpine (with or without FOLFOX). Data are shown as mean ± SEM. Statistical analysis was carried out using 1-way ANOVA with Tukey’s multiple-comparison test (A, D, and F), 2-way ANOVA with Tukey’s multiple-comparison test (B), or log-rank (Mantel-Cox) test (C). NS, not significant. Experiments were performed independently at least 3 times.
and characterized for density by measuring the weight of each fraction (g/mL, indicated in Supplemental Table 1). Fractions 1 and 2 (1.11-1.12 g/mL) were the only ones containing TEVs.

Quantitative real-time PCR. Total RNA from ECs or tumors was extracted using TRIzol reagent (Life Technologies, 15596018) and analyzed by quantitative real-time PCR using SYBR Green Master Mix (Applied Biosystems, 4367659). Primers of indicated genes are listed in Supplemental Table 2.

TEV and liposome uptake in vitro was examined in the WT and Ch25h−/− ECs cultured with TEV-free media and pretreated with vehicle or reserpine (diluted as previously described in ref. 26), followed by either DiD-labeled liposomes (FormuMax, F60103F-DD, 1 μg/mL) or TEVs that were either labeled with DiD as described previously (26) or derived from B16F10 cells stably expressing GFP. Uptake of DiD was monitored by flow cytometry as described previously (26). Uptake of Gfp mRNA was analyzed after total RNA was isolated from ECs using TRIzol reagent and chloroform. RNA concentration and purity were determined by using a NanoDrop spectrophotometer (Thermo Fisher Scientific). An Applied Biosystems High-Capacity RNA-to-cDNA Kit was used to make cDNA. The Gfp mRNA level was measured by quantitative real-time PCR.

For assessment of TEV production by tumor cells in vitro, 6 × 10⁶ cells were plated in 15-cm dishes. Upon attachment of all cells, the media were removed and replaced with fresh media that contained 10% extracellular vesicle-free FBS. The cells were treated with vehicle (DMSO) or reserpine (10 μM). After 2 days, the conditioned media were collected for extracellular vesicle isolation and the total number of cells were counted for each condition. Ten microliters of isolated extracellular vesicles was submitted for nanoparticle tracking analysis (NTA), and 10 μL was used for protein concentration. The number of vesicles per microliter and the amount of protein per vesicle were calculated correspondingly.

For assessment of TEV absolute number in plasma from tumor-bearing mice undergoing chemotherapy, 100 μL of plasma was harvested and TEVs were isolated with an exosome isolation kit (Invitrogen, 4485229). Pellets were resuspended in 50–80 μL PBS and samples were assayed by NTA.

Flow cytometric analysis of ICBT and other immunological techniques. Volume of tumors measured by caliper was calculated as width × width × length × 0.5. Tumor tissues were dissected and digested with 1 mg/mL Collagenase D (Roche, 11088882001) with 100 μg/mL DNase I (Roche, 10104159001) in RPMI medium with 2% FBS for 1 hour with continuous agitation at 37°C. The digestion mixture was passed through a 70-μm cell strainer to prepare a single-cell suspension and washed with PBS supplemented with 2 mM EDTA and 1% FBS. Single cells were stained with cell-surface antibodies: anti-CD45-APC-Cy7 (BioLegend, catalog 103115), anti-CD31-PE-Cy7 (BioLegend, catalog 102417), and anti-PDGFRα-APC (BioLegend, catalog 135907). Data were acquired using an LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay–based kits from Boster Enzyme-linked immunosorbent assay–based kits from Boster (tomato) lectin (FITC-lectin) (Thermo Fisher Scientific, L32478; 100 μg/mL) in RPMI medium with 2% FBS for 1 hour and held at 25.0 ± 0.5°C for intensity polarization measurements using a Tecan Infinite F200 Fluorescence Microplate Reader System (λem = 313 nm; λex = 460 nm). The degree of cell membrane polarization was calculated using P = (F1 − F2)/(F1 + F2), in which F1 and F2 refer to the fluorescence intensity of vertically and horizontally polarized components, respectively, with excitation vertically polarized.

**Tumorigenesis studies.** For the syngeneic subcutaneous tumor model, B16F10, MC38, or MH4499c4 tumor cells were inoculated into the right flank of indicated C57BL/6J mice (67). For xenograft studies, human HCT116 cells (5 × 10⁶) were injected into NSG mice (The Jackson Laboratory). Studies using the orthotopic colon tumor growth model were carried out as previously described (68). Briefly, after anesthesia, a 1.5-cm nick was made in the abdominal wall (around) and the cecum was exteriorized and kept moist using PBS. Twenty-five microliters of the MC38 cell suspension (2 × 10⁶/mL) was injected into the cecal wall using a 30-G needle and the injection site was covered with a cotton swab for 3 minutes to monitor for leakage. The cecum was gently returned to the abdominal wall, and then the abdominal wall and skin were sutured carefully. For the orthotopic prostate cancer model, 1 × 10⁶ TRAMP-C2-luc/GFP cells were injected into the prostate of WT or Ch25h−/− mice. Tumor volumes were tracked via detecting bioluminescence intensity weekly.

**FITC-lectin perfusion.** MC38 cells (1 × 10⁶) were injected into the right flank of WT and Ch25h−/− mice. Fourteen days after injection, mice were anesthetized, injected with FITC-conjugated Lycopersicon esculentum (tomato) lectin (FITC-lectin) (Thermo Fisher Scientific, L32478; 100 μg/mouse, i.v.) and allowed to circulate for 10 minutes. After that, the chest was opened rapidly and the vasculature was perfused with 30 mL of 4% paraformaldehyde (PFA) for 5 minutes. Tumor tissues were harvested and stored in PFA overnight before being frozen in OCT. Cryosections of tumors were stained with anti-CD31 for the whole blood vessels and FITC-positive areas were calculated with Metaphor software.
Combination therapies. Reserpine (Sigma-Aldrich, 83580) was administered as previously described (26). Briefly, reserpine (dissolved in 0.1% ascorbic acid and diluted in ddH₂O) or vehicle (0.1% ascorbic acid diluted in ddH₂O) was administered to B16F10 tumor-bearing mice when the tumors reached 75 mm³ at the dose of 1 mg/kg (i.p.; 3 times per week). Matched vehicle and reserpine mice with similar (100–130 mm³) tumor volumes were chosen to undergo irradiation using the Small Animal Radiation Research Platform (SARRP, Xstrahl Medical & Life Sciences) 2 days later. Mice were anesthetized using inhalated 2.5% isoflurane and placed on the stage of the SARRP. Once the tumor isocenter was determined, delivery of the single 12-Gy dose was made using a 1 × 1 cm collimated beam operating at 175 kV, 15 mA, with copper filtration and the dose rate at 1.65 Gy/min. The beam was delivered at such an angle as to avoid the spine. Dosimetry was performed using EBT2 gafchromic films.

The ingredients for the FOLFOX regimen (oxaliplatin, PHR1528; 5-fluorouracil, F6627; folinic acid calcium salt hydrate, F7878 — all Sigma-Aldrich) were dissolved in PBS and administered (150 mg/kg folinic acid, 5 mg/kg 5-fluorouracil, 1.4 mg/kg oxaliplatin; all i.p. every other day, with or without 1 mg/kg reserpine i.p.) into mice 10 days after the animals were inoculated with 5 × 10⁶ MC38 cells injected into the cecum. All mice were sacrificed 45 days after tumor inoculation and tumor, liver, and intestine were harvested for the histopathologic analysis. Sunitinib (BioVision, 1611) was dissolved in a vehicle (composed of carboxymethylcellulose sodium [0.5% w/v], NaCl [1.8% w/v], Tween 80 [0.4% w/v], and benzyl alcohol [0.9% w/v] in water; the whole formulation was adjusted to pH 6.0). Rebastinib was dissolved in 0.4% hydroxypropyl methylcellulose. MC38 or B16F10 cells (5 × 10⁶) were inoculated into the right flank of WT mice. Nine days after tumor inoculation, mice were treated with sunitinib (40 mg/kg, gavage) 3 times per week with or without reserpine (1 mg/kg, i.p.) every other day or rebastinib (20 mg/kg, gavage) twice per week.

RNA sequencing. Primary lung ECs from Ch25k−/− mice were pretreated with vehicle or reserpine (10 μM for 8 hours) followed by treatment with MC38 TEVs (20 μg/ml) or PBS for 12 hours in vitro and total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN) and analyzed for Angpt2 mRNA levels by qPCR. These samples were then used for RNA sequencing (carried out as previously described, ref. 69). Raw reads were mapped to the mouse reference transcriptome (Ensembl) using Kallisto version 0.46.0. Raw data are available in the NCBI’s Gene Expression Omnibus database (GEO GSE163941). All subsequent analyses were carried out using the statistical computing environment R version 4.0.0 in RStudio and Bioconductor version 3.11.1 as described in the supplemental material.

Statistics. All experiments described here are representative of at least 3 independent experiments (n > 5 mice for each group unless otherwise specified). For in vitro experiments, cells or tissues from each animal were incorporated in triplicate. All data are shown as mean ± SEM. Statistical analyses were conducted using GraphPad Prism 7 software. Comparisons between 2 groups were conducted with a 2-tailed Student’s t test and multiple comparisons were performed using 1-way ANOVA or 1-way ANOVA followed by Bonferroni’s post hoc test. Tumor growth curve analysis was conducted with repeated-measures 2-way ANOVA (mixed model) followed by Bonferroni’s post hoc test. Kaplan-Meier curves were used to analyze the survival data, and Cox regression was used to compute hazard ratios. P values of less than 0.05 were considered significant.

Study approval. Use of preexisting human archival deconvoluted and deidentified CRC tissue arrays, previously collected under informed consent, and samples that could not be directly or indirectly linked to individual human subjects was exempt from institutional review or approved by the IRB of the Medical College of Wisconsin.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with the IACUC guidelines. All mice had water ad libitum and were fed regular chow. Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines. Littermates from different cages were randomly assigned to the experimental groups. These randomized experimental cohorts were either cohoused or systematically exposed to the bedding of other groups to ensure equal exposure to the microbiota of all groups.

Author contributions SYF, ZL, HR, JAD, CK, and SWR designed the research. ZL, AO, IV, ARP, FZ, CC, PY, RMD, HZ, RK, YS, ER, ATY, EK, and DPB performed the experiments and interpreted the data. SYF, ZL, HR, JAD, CK, and SWR wrote the manuscript with the help of all authors.

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