A microRNA signature defines chemoresistance in ovarian cancer through modulation of angiogenesis

Andrea Vecchioneab,1, Barbara Bellettic, Francesca Lovatb, Stefano Volinib,d, Gennaro Chiappettab, Simona Giglioa, Maura Sonegoc, Roberto Cirombellaa, Elisa Concetta Onestia, Patrizia Pellegrinib, Daniela Califanoe, Sandro Pignataa, Simona Lositob, Vincenzo Canzonieric, Roberto Sorioc, Hansjürg Alderb, Dorothee Wernickeb, Antonella Stoppacciaroa, Maura Sonenboc, Roberto Cirombellaa, Elisa Concetta Onestia, Patrizia Pellegrinib, Daniela Califanoe, Sandro Pignataa, Simona Lositob, Vincenzo Canzonieric, Roberto Sorioc, Hansjürg Alderb, Dorothee Wernickeb, Antonella Stoppacciaro.

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Epithelial ovarian cancer is the most lethal gynecologic malignancy; it is highly aggressive and causes almost 125,000 deaths yearly. Despite advances in detection and cytotoxic therapies, a low percentage of patients with advanced stage disease survive 5 y after the initial diagnosis. The high mortality of this disease is mainly caused by resistance to the available therapies. Here, we profiled microRNA (miR) expression in serous epithelial ovarian carcinomas to assess the possibility of a miR signature associated with chemoresistance. We analyzed tumor samples from 198 patients (86 patients as a training set and 112 patients as a validation set) for human miRs. A signature of 23 miRs associated with chemoresistance was generated by array analysis in the training set. Quantitative RT-PCR in the validation set confirmed that three miRs (miR-484, -642, and -217) were able to predict chemoresistance of these tumors. Additional analysis of miR-484 revealed that the sensitive phenotype is caused by a modulation of tumor vasculature through the regulation of the VEGFB and VEGFR2 pathways. We present compelling evidence that these miRs can classify the response to chemotherapy of ovarian cancer patients in a large multicenter cohort and that one of these three miRs is involved in the control of tumor angiogenesis, indicating an option in the treatment of these patients. Our results suggest, in fact, that blockade of VEGF through the use of an anti-VEGFA antibody may not be sufficient to improve survival in ovarian cancer patients unless VEGFB signaling is also blocked.

O varian cancer is the leading cause of gynecological cancer-related death in the developed world (1). Although progress has been made in its treatment by improved debulking surgery and the introduction of platinum–taxane regimens (2), the overall 5-y survival is only 29% in advanced stage disease (1), mostly because of diagnosis at an advanced stage and intrinsic and acquired resistance to platinum-based chemotherapy. Identifying molecular markers of ovarian cancer chemoresistance is, therefore, of crucial importance. Successful translation of findings at the molecular level will lead to individualized treatment regimens, improved chemotherapeutic response rates, and avoidance of unnecessary treatments.

MicroRNAs (miRs) are a class of small noncoding RNAs that modulate gene expression by causing translational repression, mRNA cleavage, or destabilization (3). They are involved in numerous physiological cellular processes (4–7). Most importantly, accumulating evidence indicates that many miRs are aberrantly expressed in human cancers (8–10), and their expression profiles can classify stage, subtype, and prognosis of some cancers (11–14).

In this report, we describe an miR signature that defines chemoresistant ovarian carcinoma. We show that some miRs are deregulated in most patients with resistant ovarian carcinomas, and we show that miR-484 exerts its action through the regulation of angiogenic factors. We postulate that this miR signature of drug resistance could be used to develop strategies for targeted therapies in chemorefractory ovarian carcinoma patients.

Results

miR Expression in Serous Ovarian Carcinomas is Related to Chemoresis-
tance. To investigate whether miRs could predict serous ovarian carcinoma (EOC) chemoresistance, we analyzed the expression of 676 miRs in the training set (Table S1). As shown in Fig. L4, we were able to identify a response signature with 23 differentially expressed miRs capable of discrimination among the four different groups. Cluster analysis of the centroids (Fig. 1B) revealed that the EOC samples can be grouped in two major classes: complete and partial responders. Furthermore, they can be labeled as responders on one side, and stable and progressive disease, labeled nonresponders, is on the other side. Thus, we used these two classes to further refine the response signature and define 12 miRs (Fig. 1C). We used 112 EOC samples from a second patient cohort to validate the response miRs (Table S1). Of 12 miRs initially identified, we were able to confirm three miRs down-regulated in nonresponder tumors: miR-484 (P value = 0.0007), miR-642 (P value = 0.041), and miR-217 (P value = 0.046) (Fig. 1D). We focused our attention for additional studies on miR-484, which was the most statistically significant.

miR-484 Expression Does Not Alter in Vitro Sensitivity to Carboplatin and Taxol. To obtain insight into the role of miRs in the development of drug resistance, the expression levels of miR-484 were evaluated in six different epithelial ovarian carcinoma cell lines (Fig. S1A). Surprisingly, treating the cells for 2 or 4 h with increasing concentrations of carboplatin (CBDCA) and taxol (Tax) showed that their IC90 value was not related to the endogenous levels of miR-484, which were evaluated 4 d later by 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTS) assay (Fig. S1B). Moreover, overexpression of miR-484 in both MDAH-2274 and SKOV-3 cell lines did not significantly affect their in vitro sensitivity to CBDCA and Tax (Fig. S1C and D).


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1To whom correspondence may be addressed. E-mail: Andrea.Vecchione@osumc.edu or carlo.croce@osumc.edu.

2G.B. and C.M.C. contributed equally to this work.

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miR-484 was stably overexpressed in both cell lines using a lentiviral vector also encoding the EGFP protein, to better follow tumor growth in vivo. Stably transduced MDAH-2774 cells showed a 2.0- to 3.5-fold increase in the expression of the miR compared with scr-transduced cells shown in Fig. 5A, differences similar to those observed in primary EOC between responders and nonresponders in human and mouse tumors (114) for nonresponders (mean micrometastases: 120 ± 890 for SKOV-3-miR-484, 23 ± 114 for scramble-transduced tumors), whereas scramble-transduced tumors showed 12% ± 17 (range = 0–65) of necrosis.

Role of miR-484 in the Acquisition of Chemoresistance. Because levels of miR-484 were similar among cell lines, we used MDAH-2774 and SKOV-3 cells overexpressing either control miR (scr) or miR-484 to create, in vivo, the responder phenotype and explore the possibility that miRs influence the chemosensitivity of ovarian cancer in a context-dependent manner. miR-484 was stably overexpressed in both cell lines using a lentiviral vector also encoding the EGFP protein to better follow tumor growth in vivo. Stably transduced MDAH-2774 cells showed a 2.0- to 3.5-fold increase in the expression of the miR compared with scr-transduced cells shown in Fig. 5A, differences similar to those observed in primary EOC between responders and nonresponders in human and mouse tumors. In vitro studies using a fluorescence microscope showed that the EGFP fluorescence was scarcely detectable in MDAH-2774 cells (gray value: 750 ± 120 and 850 ± 230 for MDAH-2774 miR-484 and scr, respectively), whereas it was very bright in SKOV-3 cells, with miR-484 cells slightly brighter than scr-transduced cells (gray value: 5,450 ± 890 and 3,250 ± 630 for SKOV-3 miR-484 and scr, respectively). For these reasons, only SKOV-3 cells were followed for their growth using an in vivo imaging system able to detect EGFP fluorescence, whereas the growth of MDAH-2774 miR-484 and scr-transduced cells was followed using a caliper-based method. Cells (1.5 × 10⁶) were inoculated into nude mice s.c. into the left (EGFP-miR-484-MDAH-2774) and right (EGFP-MDAH-2774) flanks and allowed to grow for 15 d. At this time, the tumor volume formed by miR-484-expressing cells was not statistically different from the one observed in control EGFP-expressing cells (Fig. 2A, Left), indicating that the growth of the primary tumor, at least in the first phases of tumor development, was not affected by the expression of the miR. After CBDCA and Tax treatment, control tumors increased their size about sixfold compared with day 0 at the end of the treatment (range = 2.3–17.7). In the same mice, miR-484–expressing tumors increased only 1.3-fold (range = 0.8–1.8), showing to be more sensitive to the drugs than the controls (Fig. 2A, Right). Using an in vivo imaging system able to detect EGFP fluorescence, the analysis of SKOV-3 cells confirmed that the expression of miR-484 did not affect the growth of the primary tumor but significantly increased the sensitivity to treatment (Fig. 2 B and C). The higher fluorescence intensity of tumors expressing miR-484 in untreated mice (Fig. 2C, Left) was in fact, expected, and it reflected the higher EGFP fluorescence intensity of miR-484-SKOV-3 cells observed in vitro. We then used MDAH-2774 cells, which grew more rapidly in nude mice than SKOV-3 cells, to examine whether in vivo administration of the miR could alter the sensitivity of EOC to CBDCA+Tax treatment. Parental MDAH-2774 cells were allowed to grow for 2 wk in the flanks of nude mice, and then, they were injected with lentivirus-expressing EGFP-control miR in the right flank and lentivirus-expressing EGFP-miR-484 in the left flank. Beginning 2 d later, mice were treated biweekly for 3 wk with CBDCA+Tax, and the intratumoral injection of virus was repeated after 1 wk. After 21 d, tumor growth was evaluated. Strikingly, in six of six cases, miR-484 increased drug sensitivity, showing that its expression is able to modulate resistance to CBDCA and Tax in epithelial ovarian cancer in vivo (Fig. 2D). To examine the tumor response, mouse samples were stained with H&E, and apoptosis was evaluated by TUNEL assay. Although no increase in apoptosis was observed, it was noted that, in miR-484–transduced tumors, there was a dramatic increase in necrosis. In fact, as shown in Fig. 2E and F, miR-484–transduced tumors showed 58.4% ± 17 (range = 27–92), whereas scramble-transduced tumors showed 12% ± 17 (range = 0–65) of necrosis.

miR-484 Regulates the Expression of Angiogenic Factors. Our data pointed to a role of miR-484 in the chemosensitivity of EOC cells in vivo but not in vitro, suggesting that it might act on the tumor microenvironment rather than in a cell autonomous manner. Searching for putative targets, we found that miR-484 may be involved in the regulation of angiogenic factors. In fact, miR-484, according to different prediction algorithms, targets the VEGFB, which is able to directly stimulate endothelial cell growth and migration (15), and the VEGFR2/KDR, which is implicated in all aspects of normal and pathological vascular endothelial cell biology (16). Luciferase and Western blot analyses confirmed that miR-484 overexpression down-regulated the endogenous levels of VEGFB and VEGFR2 (Fig. 3). Thus, we hypothesized that some modifications in the vascular asset of responders and nonresponders in human and mouse tumors might occur. Vascular density was assessed using an antibody against anti-CD34, which is a single-chain transmembrane glycoprotein, associated with human hematopoietic progenitor cells. We used 30 cases of human serous ovarian carcinoma (15 responders and 15 nonresponders) and 28 cases of mice xenograft tumors [14 cases from SKOV-3 and 14 cases from MDAH-2774 (7 cases transduced with miR-484 and 7 cases transduced with EGFP)] and evaluated using the Chalkley eyepiece method (17). In human tumors, the mean microvessel density was 30 ± 1.17 (range = 9–54) for responders and 68 ± 1.6 (range = 15–114) for nonresponders (P = 0.0000002); in mice, it was 9 ± 5.5 (range = 1–18) for SKOV-3-miR-484, 23 ± 12 (range = 5–37) for SKOV-3-EGFP (P = 0.0004), 10 ± 6.2 (range = 4–22) for
MDA-H-2774-miR-484, and 17 ± 8.9 (range = 5–28) for MDAH-2774-EGFP (P = 0.0009) (Fig. 4A and B). Performing a Spearman rank correlation test and correlating miR-484 expression values and microvessel density of the same samples, we found a strong inverse relationship between vessel number and miR expression (r = −0.8, R = 1.56E−07), suggesting that the sensitivity of these tumors is caused by their microvessel asset driven by miR regulation (Fig. 4A). Accordingly, the expression of VEGFB and VEGFR2 in human tumors correlated with the different levels of miR-484 and the response to therapy. Indeed, responder tumors (high miR-484) showed levels of both VEGFB and VEGFR2 significantly lower than nonresponder tumors (Fig. 4C and D) as evaluated by immunohistochemistry. Among 15 responder tumors, 13 tumors expressed weak levels of VEGFB, and 11 tumors expressed weak levels of VEGFR2 (Fig. 4C, Right and D, Right). Conversely, in nonresponder tumors, all but one tumor expressed moderate/high levels of both VEGFB and VEGFR2 (Fig. 4C, Right and D, Right).

miR-484 Is a Secreted miR That Regulates the Expression VEGFR2 in Tumor-Associated Endothelial Cells. Our data indicated, so far, that miR-484 is involved in the regulation of both VEGFB and VEGFR2 in primary ovarian cancers and xenograft tumors formed by ovarian cancer cells (Fig. 4). Although these data compellingly showed that miR-484 targeted VEGFB in EOC cells, we did not observe any effects of miR-484 on VEGFR2 expression in EOC cells, mostly because all of the tested cell lines expressed very low/undetectable levels of the receptor (Fig. 5E).

It is now well-accepted that miRs can be secreted and/or released in the local microenvironment and into circulation, acting as paracrine and/or endocrine regulators of several biological functions, including cancer cell growth (18). We, thus, hypothesized that miR-484 could be released into the local microenvironment from EOC cells and penetrate into the endothelial cells, thus eventually modulating its targets. To prove this hypothesis, we first evaluated if miR-484 could be secreted into the conditioned medium of EOC. We evaluated the levels of miR-484 in MDAH-2774 and SKOV-3 cells stably transduced with scr or miR-484 lentiviral vectors in their conditioned medium (CM). Normalized expression of the miR-484 revealed that stably transduced cells showed a two- to threefold increase of its expression over controls (Fig. 5A). Importantly, the levels of miR-484 in the CM of SKOV-3 and MDAH-2774 exactly paralleled the miR levels observed in the cells (Fig. 5B), showing that miR-484 is secreted and that the amount of miR secreted is directly dependent on the amount of miR-484 produced by cancer cells. Next, we asked whether the secreted miR could penetrate into tumor-associated endothelial cells. We cocultured human umbilical vein endothelial cells (HUVEC) and ovarian cancer cell-derived cell lines overexpressing miR-484-EGFP or scrambled miR-EGFP using a transwell-based assay. HUVEC cells, thus eventually modulating its targets. To prove this hypothesis, we first evaluated if miR-484 could be secreted into the conditioned medium of EOC. We evaluated the levels of miR-484 in MDAH-2774 and SKOV-3 cells stably transduced with scr or miR-484 lentiviral vectors in their conditioned medium (CM). Normalized expression of the miR-484 revealed that stably transduced cells showed a two- to threefold increase of its expression over controls (Fig. 5A). Importantly, the levels of miR-484 in the CM of SKOV-3 and MDAH-2774 exactly paralleled the miR levels observed in the cells (Fig. 5B), showing that miR-484 is secreted and that the amount of miR secreted is directly dependent on the amount of miR-484 produced by cancer cells. Next, we asked whether the secreted miR could penetrate into tumor-associated endothelial cells. We cocultured human umbilical vein endothelial cells (HUVEC) and ovarian cancer cell-derived cell lines overexpressing miR-484-EGFP or scrambled miR-EGFP using a transwell-based assay. HUVEC expressed low levels of miR-484, which were slightly increased by coculture with scrambled miR-EGFP–expressing cells when both SKOV-3 and MDAH-2774 cells were used (Fig. 5C). However, levels of miR-484 increased from one- to fivefold compared with controls in HUVEC cells cultured in the presence of...
Collectively, these data show that miR-484 produced from EOC cells is secreted by the neoplastic cells into the local microenvironment and enters HUVEC cells within 24 h. The passage of miR-484 from EOC to endothelial cells had functional consequences: in fact, when HUVECs were cocultured with control and miR-484-overexpressing SKOV-3 cells for 24 h, only the latter cells were able to significantly decrease the expression of VEGFR2 protein on endothelial cells (Fig. 5E).

Overall, in vitro and in vivo data show that miR-484 targets both VEGFB and VEGFR2 proteins and suggest that the expression of miR-484 in EOC cells could influence endothelial cell growth and motility in a paracrine manner. To prove this hypothesis, the CM from MDAH-2774 or SKOV-3 cell lines

miR-484–overexpressing cell lines (Fig. 5C). In addition, the level of miR-484 in HUVEC cells paralleled the expression of miR-484 in cocultured endothelial cells (compare Fig. 5A with Fig. 5C). Because the coculture was performed using a transwell-based assay, in which cancer and endothelial cells were separated by a porous membrane, we could exclude that a direct contact between ovarian carcinoma cell lines and HUVEC cells was necessary for the passage of miR-484 from cancer to endothelial cells. Accordingly, incubating HUVEC cells for 24 h only with the CM from miR-484-overexpressing SKOV-3 cells resulted in a twofold increase in the levels of miR-484 compared with controls. To further prove that miR-484 could pass from EOC to endothelial cells when the two types of cells were cocultured, we transfected SKOV-3 cells with a fluorescently labeled miR-484 oligo; 12 h later, cells were washed, detached, and replated in a 1:1 dilution with HUVEC and then cocultured for an additional 24 h. At this time point, cells were fixed and stained with the anti-CD31 antibody to identify the cocultured endothelial cells. As shown in Fig. 5D, not only did all of the CD31-negative SKOV-3 cells show the presence of labeled miR-484 in their cytoplasm, but also, $38 \pm 10\%$ of CD31-positive endothelial cells were positive for the fluorescently labeled miR-484 (Fig. 5D).
paired when CM from MDAH-2774 able to induce the formation of tube-like structures when HUVEC cells were cultured in the presence of CM for 20 h (Fig. S2).

The fact that ovarian cancer cells overexpressing miR-484 are less able to stimulate endothelial cell reorganization in vitro (Fig. S2) was used and abolished when three miRs (484, 642, and 217) were capable of conferring a responder or nonresponder status on ovarian cancer. Focusing on the validation set (112 samples) using the TaqMan MicroRNA assay. Differentially expressed miRs were validated on the validation set (112 samples) using the TaqMan Low-Density Array (TLDA) cards and RT-PCR data performed on the training set (86 samples) using TaqMan Array Human MicroRNA Set v2.0 containing a total of 676 unique assays. Differentially expressed miRs were validated on the validation set (112 samples) using the TaqMan MicroRNA assay.

Materials and Methods
A more detailed and complete description of all methods is provided in SI Materials and Methods.

Discussion
Considering the poor prognosis for patients with ovarian neoplasms, mainly because of late diagnosis and low response to chemotherapy, we have attempted to identify predictive markers of therapeutic response and molecular targets to increase sensitivity to treatment. miRs, a class of gene regulators, have been proven to be effective in classifying normal and cancerous tissues as well as cancer prognosis. Data on ovarian cancer thus far indicate that the miR network is very important to understanding ovarian cancer biology and resistance to therapy (19, 20). Analyzing 198 samples of serous ovarian carcinoma, we discovered that three miRs (484, 642, and 217) were capable of conferring a responder or nonresponder status on ovarian cancer. Focusing our attention on miR-484, we unveiled its mechanism of action in modulating chemosensitivity through the vasculature asset. It is interesting to note that this miR targets both VEGF signaling pathways by either directly modulating the VEGFB protein on the neoplastic cells or interfering with the receptor VEGFR2 in the tumor-associated endothelial cells. The concomitant modulation of the VEGFB and VEGFR2 leads to a normalization of the tumor microenvironment through the control of new vessel formation and maturation, thus improving cancer treatment (21, 22).

Several reports point to the VEGFB–VEGFR1 axis as one of the determinants of ovarian cancer neoangiogenesis (23, 24).

The ovarian cancer cells overexpressing miR-484 are less able to stimulate endothelial cell reorganization in vitro (Fig. S2) and neoangiogenesis in mice (Fig. 4) strongly supports this hypothesis. It is also likely that the cooperation between VEGFR1 and -2 signaling is necessary for ovarian cancer growth and drug sensitivity. Indeed, recent results showed that only the simultaneous inhibition of VEGFR1 and -2 is able to significantly reduce the growth of solid human ovarian carcinoma injected i.p. in mice (25).

The importance of neoangiogenesis in ovarian cancer has been proven in human patients, where several phase II clinical trials with antiangiogenic compounds used as single agents to pretreat women resulted in a 16–21% response rate (26). These trials were instrumental for the randomized phase III studies [gynecologic oncology group (GOG) 218 and ICON-7] that compared the added use of bevacizumab with the standard i.v. agents carboplatin and paclitaxel. The results from GOG 218 showed an additional 3.8 mo of progression-free survival (27), whereas the ICON-7 study reported an increased progression-free survival of 1.7 mo (28). Interestingly, the ICON-7 study showed that the benefit of adding bevacizumab to standard chemotherapy particularly benefited patients at high risk of disease progression, supporting our hypothesis that targeting tumor neoangiogenesis will represent a useful approach in the treatment of ovarian cancer patients. However, adding bevacizumab to standard treatment will not represent a cost-effective option for advanced ovarian cancer patients (27). Furthermore, even tumors initially responding to anti-VEGF/VEGFR2 therapy ultimately acquire resistance to the treatment, and relapse occurs in virtually all patients (29). Thus, although antiangiogenic therapy has proven to be a valuable tool in cancer treatment, there is an urgent need for alternative strategies to target the tumor vasculature and better select the patients who will benefit from antiangiogenic compounds. Our work provides important information on both of these aspects and will likely lead to an improved treatment for ovarian cancer patients. In fact, we discovered that the molecular signature of three miRs could potentially identify those patients who will respond to conventional chemotherapy and those patients who will effectively benefit from the addition of antiangiogenic compounds, thereby also reducing the costs of the therapies and improve the efficacy of the drugs. Moreover, our data strongly suggest that blockade of VEGF by the use of an anti-VEGFA antibody alone may not be useful in ovarian cancer patients unless VEGFB signaling is also blocked. Alternatively, small compounds, such as functionalized nanoparticles (30) targeting the VEGFR1 and -2 receptors, could be used as effective therapeutic agents in these patients, changing the course of prognosis and treatment of ovarian cancer.
quantification of gene expression on Data Assist ver.1.2 (Applied Biosystems).

For each sample, the mean miR expression value was calculated as the average of Ct values smaller than 35. Samples were labeled based on either their response to first chemotherapy or other clinical parameters. We used the one-way ANOVA test to identify differentially expressed miRs using R software. Global median normalization was used for the expression analysis of the TLDA cards. miR-16 and -191, both among the most invariable miRs in the training set, were used as endogenous controls for normalization of the RT-PCR in the validation set. All data were expressed as the mean ± SEM. Statistically significant differences between nonresponders and responders


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Supporting Information

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SI Materials and Methods

**RNA Extraction.** Total RNA for low-density array and single array analyses was isolated from paraffin-embedded tissue (FFPE) using the High Pure FFPE RNA Micro Kit (Roche Applied Science) according to the manufacturer’s protocol. Total RNA was quantified using NanoDrop (Thermo Fisher Scientific Inc), and the presence of small RNAs was checked with a 2100 Bioanalyzer (Agilent).

**MicroRNA Expression Profiling Using TaqMan Array Human MicroRNA Set Cards v2.0.** MicroRNA (miR) expression profiling was performed on the training set (86 samples) using TaqMan Array Human MicroRNA Set v2.0, a preconfigured 2-microfluidic card set, A and B, containing 676 unique assays specific to human miRs and four control assays (three selected candidate endogenous control assays and one negative control assay). All sample concentrations were adjusted to the lowest limiting sample (15 ng/µL). To increase sensitivity, a preamplification step was included using Megaplex PreAmp Primers Human Pool Set v2.0. For miR cDNA synthesis, RNA was reverse-transcribed using the miR reverse transcription kit (Applied Biosystems) combined with the stem-loop Megaplex Primer Pools according to the manufacturer’s protocol. All PCR reactions were performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Raw Ct values were calculated using the SDS software v.2.1 using automatic baseline settings and a threshold of 0.2.

**TaqMan Array Human miR Set Cards v2.0 Data Validation and Real-Time PCR for Mature miRs in Cell Lines.** Differentially expressed miRs were validated on the validation set (112 samples) using the TaqMan Single-Tube MicroRNA Assays. For cell lines, total RNA was isolated with TRIzol (Invitrogen). All reagents, primers, and probes were obtained from Applied Biosystems. Normalization of the validation set was performed with miR-16 and -191, two of the most invariant miRs in the training set. Reverse transcriptase reactions and real-time PCR were performed according to the manufacturer’s instructions. All reverse transcriptase reactions and real-time PCR were performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method.

**In Vitro Susceptibility to Carboplatin + Taxol Treatment of Ovarian Carcinoma Cell Lines Expressing miR-484.** To determine the IC₅₀ values of ovarian cancer-derived cell lines, MADH-2774, SKOV-3, TOV21G, TOV112D, OVCAR8, and IGROV were plated in 96-well plates (1,000 cells/well) and treated with the indicated doses of CBDCA and Taxol. Viability was evaluated after 4 d as described above.

**Lentivirus Expression Using TaqMan Array Human MicroRNA Set Cards v2.0.** The same was carried out with SKOV-3, except that 2.2 × 10⁶ cells were injected. When tumor masses became visible, treatment with CBDCA (15 mg/kg) and Tax (5 mg/kg) was started. Drugs were delivered diluted in 200 µL PBS and i.p. injected two times per week for 4 wk. For intratumoral injection experiments, mice (n = 8) were s.c. injected on both dorsal flanks with 100 µL PBS containing 1.5 × 10⁶ MADH-2774 cells and miR-484 (right flank) or miR-484 (left flank). The same was carried out with SKOV-3, except that 2.2 × 10⁶ cells were injected. When tumor masses became visible, 50 µL lentiviruses (scrambled-miR in the right flank or miR-484 in the left flank) in PBS containing 1.5 × 10⁶ TU were injected into tumors through a 30-gauge needle two times during the first week of treatment with drugs (same as above). Tumor masses were measured with a caliper two times per week for 4 wk. Moreover, transduced cells exploiting the expression of EGFP tumor masses have been imaged by the IVIS Lumina (Caliper) one time per week for 4 wk. Mice were then killed, and tumor masses were excised and included in Tissue-Tek OCT (Sakura) for additional analyses.

**TUNEL Assay and Necrosis Evaluation.** TUNEL assay was performed on frozen tumor tissues according to the manufacturer’s instructions (Roche). For necrosis evaluation, mouse tumors were stained in H&E, and slides were scanned at 20x magnification using the Aperio SCANSCOPE CS SYSTEM. After the acquisitions, images were analyzed for tumor volume and area of necrosis using Imagescope software.

**miR Overexpression and Western Blot.** One hundred nanomolar premiR-484 and prenegetative control-2 (Ambion) were transfected into SKOV-3 and HUVEC cells using Lipofectamine 2000 (Invitrogen). After 24 h, cells were harvested, and Western blot analysis was performed using the following primary antibodies: rabbit polyclonal anti-VEGFR2 (1:1,000; Cell Signaling Technology), mouse monoclonal anti-VEGFb (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-α-tubulin (1:2,000; Sigma), and mouse monoclonal antivinculin (1:2,000; Santa Cruz Biotechnology).

**Luciferase miR Target Reporter Assay.** The 3’ UTRs of the human VEGFR2, and VEGFB genes were PCR-amplified (primers are available on request) and inserted into pGL3 control vectors (Promega) using the Xba1 site immediately downstream from the stop codon of Firefly luciferase, giving rise to the p3’ UTR-VEGFR2 and p3’UTR-VEGFB plasmids. These constructs were used to generate the p3’-UTRmut-VEGFR2 and
p3'-UTRmut-VEGFB plasmids using a QuikChange site-directed mutagenesis kit (Stratagene).

SKOV-3 cells were cotransfected with 1 μg p3'UTR-VEGFR2 or p3'UTR-VEGFB, p3'UTRmut-VEGFR2 or p3'UTRmut-VEGFB plasmids, 0.1 μg Renilla luciferase expression construct pRL-TK (Promega), and 100 nM miR or control precursors using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h posttransfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer’s instructions. Three independent experiments were performed in triplicate.

**Immunohistochemistry, Assessment of Vascular Density, and Immuno-fluorescence.** Tumor sections (2-μm thick) were cut, formalin-fixed, and paraffin-embedded; sample deparaffinization was achieved through graded alcohols. The primary antibodies were CD34 mouse monoclonal (1:50; Dako), VEGFB mouse monoclonal (clone 58013, 1:20; R&D), and VEGFR2 rabbit (9698, 1:1,000; Cell Signaling). All antibodies were used according to the manufacturer’s instruction. Vascular density (CD34+), expressed as the mean value of three counts, was assessed using the Chalkley eyepiece method as previously reported (1).

Positive VEGFB and VEGFR2 immunostaining was defined as a cytoplasmic and/or membranous staining of tumor cells and scored according to a three-tiered system (1, weak staining; 2, moderate staining; 3, strong staining). All sections were stained in a single run to avoid procedural variations affecting the credibility of comparing VEGF intensity readings.

For immunofluorescence experiments, SKOV-3 cells were plated into 12-well plates, cultured for 24 h, and transfected with 20 pmol miR-484–fluorescein-conjugated (miRCURY LNA; Exiqon) with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instruction. Twenty-four hours after transfection, cells were trypsinized, rinsed with PBS, and cosedeed on coverslips with HUVEC cells for 24 h. Cells were then washed two times with PBS, fixed in 3.7% (vol/vol) paraformaldehyde for 20 min at 25 °C, and permeabilized for 2 min with 0.05% Triton X-100 in PBS at 25 °C. After washing three times with PBS, cells were blocked with 1% BSA (Sigma Chemical) in PBS containing 0.05% Tween-20 for 30 min and incubated with monoclonal anti-CD31 (DAKO) for 1 h at 25 °C followed by goat anti-mouse IgG–TexasRed (Jackson Immunoresearch Laboratories) for 30 min at 25 °C. DNA was stained with DAPI. Fluorescence signals were analyzed using an Axiovert 200 inverted microscope with an ApoTome System (Zeiss).

**Tube Formation Assay on HUVEC Cells Stimulated with Conditioned Media Harvested from Ovarian Carcinoma Cell Lines Expressing miR-484.** A 48-well plate was filled with 125 μL Matrigel (nongrowth factor-reduced, 8–10 μg/μL, BD) and allowed to solidify at 37 °C for 1 h. HUVEC cells (1 × 10^5) resuspended in 200 μL medium were then layered onto the Matrigel and monitored over time for the formation of tube-like structures. Time-lapse video microscopy (Leica) was used to create a movie of this process, and it collected images every 5 min for up to 20 h. Media used to resuspend endothelial cells were (i) complete medium for endothelial cells (used as positive control), (ii) serum-reduced medium (2%; used as negative control), and (iii) conditioned medium (plus 2% serum) from SKOV-3 or MDAH-2774 transduced with scrambled or miR-484.

**Coculture of HUVEC and Ovarian Carcinoma Cell Lines Expressing or Not Expressing miR-484.** For coculture experiments, control or miR-484 MDAH-2774– and SKOV-3–overexpressing cells were plated in a 24-well plate and HUVEC cells on Transwell insert (Costar). One day later, the Transwell covered with HUVEC cells was added to the well containing the serous ovarian carcinoma cells and incubated for 24 h at 37 °C. HUVEC and serous ovarian carcinoma cells were then separately collected, and mRNA and proteins were extracted for Western blot and quantitative RT-PCR analyses as described above.

**Real-Time PCR in Conditioned Medium.** miRs from medium samples were isolated with TRIzol LS reagent (Invitrogen) in accordance with the manufacturer’s instructions for liquid samples. After DNase treatment (Roche), RNA concentrations were determined with NanoDrop (Thermo Scientific). All quantitative RT-PCRs were performed with Brilliant II QRT-PCR AffinityScript Two-Step (Stratagene). Real-time PCR was carried out on an MX3500P QPCR System (Agilent Technologies). Mature miRs were assayed using the Taq-Man MicroRNA Assays in accordance with the manufacturer’s instructions (Applied Biosystems). Samples were normalized with spike-in control using synthetic Caenorhabditis elegans miR (cel-miR-39) that was added to each sample (miRVan miR mimic; Applied Biosystems). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method.

**Fig. S1.** (A) Expression of miR-484 in ovarian cancer-derived cell lines. (B) Table shows the normalized expression levels of miR-484 in the indicated ovarian cancer cell lines and their IC$_{50}$ values for CBDCA treatment evaluated as described in Materials and Methods. (C and D) Effects of miR-484 expression on the in vitro sensitivity of (C) MDAH-2274 and (D) SKOV-3 cells to (Upper) CBDCA and (Lower) Tax treatments.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>miR-484</th>
<th>CBDCA</th>
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<tbody>
<tr>
<td>IGROV</td>
<td>0.27</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>MDAH2774</td>
<td>0.32</td>
<td>40µg/ml</td>
</tr>
<tr>
<td>OVCAR8</td>
<td>0.13</td>
<td>10µg/ml</td>
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<tr>
<td>TOV112D</td>
<td>0.28</td>
<td>15µg/ml</td>
</tr>
<tr>
<td>TOV21G</td>
<td>0.18</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>0.31</td>
<td>50µg/ml</td>
</tr>
</tbody>
</table>
**Fig. S2.** Typical images of HUVEC incubated on 3D Matrigel Matrix for 20 h in the presence of the indicated conditioned medium (CM) or controls. As shown, CM from SKOV-3– and MDAH-2774–transduced miR-484 cells reduces the ability of HUVEC to properly form and maintain tube-like structures in 3D compared with CM form control cells.

### Table S1. Patient data of training set (n = 86) and validation set (n = 112)

Baseline characteristics of 198 patients with invasive serous ovarian carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Training (86 patients)</th>
<th>Validation (112 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>Mean</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>Range</td>
<td>19–83</td>
<td>24–80</td>
</tr>
<tr>
<td>Tumor grade no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (1–2)</td>
<td>20 (23)</td>
<td>18 (15)</td>
</tr>
<tr>
<td>High (3)</td>
<td>66 (77)</td>
<td>94 (85)</td>
</tr>
<tr>
<td>Tumor stage no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I or II</td>
<td>18 (21)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>III or IV</td>
<td>69 (79)</td>
<td>105 (94)</td>
</tr>
<tr>
<td>Surgery outcome no. (%)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>25 (29)</td>
<td>55 (49)</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>40 (46)</td>
<td>28 (25)</td>
</tr>
<tr>
<td>No surgery</td>
<td>21 (25)</td>
<td>29 (26)</td>
</tr>
<tr>
<td>Response to chemotherapy no. (%)†</td>
<td>36 (42)</td>
<td>48 (43)</td>
</tr>
<tr>
<td>Complete response</td>
<td>36 (42)</td>
<td>48 (43)</td>
</tr>
<tr>
<td>Partial response</td>
<td>12 (14)</td>
<td>26 (23)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>10 (12)</td>
<td>7 (6)</td>
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<tr>
<td>Progressive disease</td>
<td>28 (32)</td>
<td>32 (28)</td>
</tr>
</tbody>
</table>

*Optimal cytoreduction is defined as a residual tumor of 1 cm or less in diameter.
†According to RECIST guideline.

Patients were all treated with CBDCA+Tax.
Movie S1. HUVEC tube formation assay in the presence of conditioned medium (CM) from scramble (scr) transduced MDAH-2274 cells. HUVEC were plated on Matrigel matrix and cultured in the presence of CM from scr-MDAH-2274 supplemented with 2% FBS. Tube formation was followed by video-time-lapse microscopy. Images were collected every 5 min for 6 h and used to create a video (15 images per s) using a dedicated software (AF6000LX; Leica). In the video, it is possible to appreciate that CM from scr-MDAH-2274 cells was able to sustain the formation of tube-like structures by HUVEC.

Movie S2. HUVEC tube formation assay in the presence of CM from miR-484 transduced MDAH-2274 cells. HUVEC were plated on Matrigel matrix and cultured in the presence of CM from miR-484-MDAH-2274 supplemented with 2% FBS. Tube formation was followed by video-time-lapse microscopy. Images were collected every 5 min for 6 h and used to create a video (15 images per s) using a dedicated software (AF6000LX; Leica).