VEGFA activates an epigenetic pathway upregulating ovarian cancer-initiating cells

Kibeom Jang\textsuperscript{1,2}, Minsoon Kim\textsuperscript{1,2}, Candace A Gilbert\textsuperscript{1}, Fiona Simpkins\textsuperscript{1,3,\dagger}, Tan A Ince\textsuperscript{1-4,5,\dagger\dagger} & Joyce M Slingerland\textsuperscript{1,2,6,\ast,\dagger\dagger}

Abstract

The angiogenic factor, VEGFA, is a therapeutic target in ovarian cancer (OVCA). VEGFA can also stimulate stem-like cells in certain cancers, but mechanisms thereof are poorly understood. Here, we show that VEGFA mediates stem cell actions in primary human OVCA culture and OVCA lines via VEGFR2-dependent Src activation to upregulate Bmi1, tumor spheres, and ALDH1 activity. The VEGFA-mediated increase in spheres was abrogated by Src inhibition or SRC knockdown. VEGFA stimulated sphere formation only in the ALDH1\textsuperscript{+} subpopulation and increased OVCA-initiating cells and tumor formation in vivo through Bmi1. In contrast to its action in hematopoietic malignancies, DNA methyl transferase 3A (DNMT3A) appears to play a pro-oncogenic role in ovarian cancer. VEGFA-driven Src increased DNMT3A leading to miR-128-2 methylation and upregulation of Bmi1 to increase stem-like cells. SRC knockdown was rescued by antagonir to miR-128. DNMT3A knockdown prevented VEGFA-driven miR-128-2 loss, and the increase in Bmi1 and tumor spheres. Analysis of over 1,300 primary human OVCAs revealed an aggressive subset in which high VEGFA is associated with miR-128-2 loss. Thus, VEGFA stimulates OVCA stem-like cells through Src-DNMT3A-driven miR-128-2 methylation and Bmi1 upregulation.

Keywords Bmi1; epigenetic; miR-128; ovarian cancer stem cell; VEGF; VEGFA

Subject Categories Cancer; Chromatin, Epigenetics, Genomics & Functional Genomics; Urogenital System

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Introduction

Developing cancers rapidly outstrip the diffusion capacity of nutrients and oxygen and must form new blood vessels via angiogenesis (Bergers & Benjamin, 2003). They must also maintain self-renewal capacity despite adverse conditions of suboptimal pH, nutrient, and oxygen availability. Vascular endothelial growth factor A, VEGFA, is a key angiogenic factor that alters the endothelial cell niche to promote new vessel formation (Bergers & Benjamin, 2003). In various cancers, VEGFA stimulates not only angiogenesis but also tumor growth, metastasis, and survival (Wu et al, 2006; Hu et al, 2007; Paez-Ribes et al, 2009).

The importance of VEGFA in angiogenesis and its frequent upregulation in human cancers (Goel & Mercurio, 2013) stimulated development of VEGF- and VEGF receptor-targeted therapies. The monoclonal antibody, bevacizumab, blocks VEGFA interaction with receptors VEGFR1 and 2 (Ferrara, 2004). Despite initial promise, VEGF-targeted therapies have shown limited efficacy, with short responses in most solid tumors (Eskander & Tewari, 2014). Hypoxia resulting from inhibition of angiogenesis upregulates VEGFA expression, contributing to aggressive disease recurrence and angiogenic therapy failure (Ebos et al, 2009; Paez-Ribes et al, 2009). In ovarian cancer, bevacizumab significantly increases progression-free survival (PFS) compared with chemotherapy alone in advanced disease, and more recently VEGFR-targeting agents were shown to significantly increase PFS (Eskander & Tewari, 2014) and overall survival (OS; Witteveen et al, 2013). Bevacizumab is approved for use with non-platinum chemotherapy in Europe and the USA for platinum-resistant ovarian cancer; however, responses are of short duration and resistance rapidly emerges (Eskander & Tewari, 2014).

Increasing evidence indicates that cancer stem-like cells (CSCs) comprise a distinct self-renewing subpopulation that can generate cancerous progeny with reduced replicative potency (Dalerba et al, 2007). CSCs are important therapeutic targets: they may not only initiate tumors but also mediate recurrence and metastasis (Magee et al, 2012). Most anticancer drugs kill the bulk cancer population.
CSCs either proliferate too slowly for targeting by cycle active drugs, or escape chemotherapy by drug expulsion or greater DNA repair, leading to recurrence (Magee et al., 2012). CSCs share properties with normal tissue stem cells, expressing discrete surface markers and forming spheres when seeded at single cell density (Visvader & Lindeman, 2012). Subpopulations of many malignancies, including OVCA, with aldehyde dehydrogenase 1 activity (ALDH1+) are enriched for CSC properties in vitro and the ability to initiate tumors in immunocompromised mice (Ginestier et al., 2007; Landen et al., 2010). Several surface markers have been proposed to characterize OVCA stem-like cells (Shah & Landen, 2014). The ALDH1+ subpopulation of primary ovarian cancers (Stewart et al., 2011) and in OVCA lines (Silva et al., 2011; Shah & Landen, 2014) is enriched in tumor-initiating cells in vivo and by prior chemotherapy exposure (Landen et al., 2010). While certain cytokines increase CSC and enhance tumor initiation in vivo (Zhao et al., 2014), extracellular growth factors and signaling pathways that stimulate CSC expansion are poorly characterized. A greater understanding of pathways governing CSC may permit the design of more effective anticancer treatments.

VEGFA is not only a potent angiogenic factor, it also stimulates stem-like cells in both normal and cancer tissues. VEGFA maintains normal stem cell populations in hematopoietic (Gerber et al., 2002), endothelial (Kane et al., 2011), and neuronal tissues (Calvo et al., 2011). VEGFA was recently shown to increase tumor-initiating stem-like cells in skin and breast cancers (Beck et al., 2011; Goel et al., 2013; Zhao et al., 2014). Pathways activated by VEGFA that increase cancer stem-like cells and tumor initiation are largely uncharacterized. Since VEGFA is frequently overexpressed in OVCA (Yu et al., 2013) and VEGF/VEGFR-targeted therapies have significant activity in this cancer (Eskander & Tewari, 2014), we investigated whether VEGFA drives ovarian CSC expansion and sought to identify targetable pathways mediating these effects.

MicroRNAs (miRNAs) are increasingly implicated in CSC regulation (Takahashi et al., 2014). These small non-coding RNAs bind the 3’ untranslated region (3’ UTR) of target genes to inhibit gene expression. miRNAs regulate targets essential for normal and malignant stem-like cell self-renewal (Takahashi et al., 2014) and are often misregulated in cancer (Croce, 2009). Oncogenic miRNAs target tumor suppressors and increase drug resistance and metastasis. In contrast, tumor suppressor miRNAs are frequently downregulated in cancer (Croce, 2009). Here, we investigated the role of VEGFA as a driver of stem-like cell expansion in OVCA. This work reveals a novel pathway linking VEGFA to miRNA-dependent CSC regulation. We show that VEGFA activates Src and induces DNMT3A to methylate miR-128-2, leading to increased Bmi1 and OVCA stem-like cell expansion.

Results

VEGFA increases sphere formation and ALDH1 activity in OVCA populations

While VEGFA is an angiogenic agent and therapeutic target in OVCA, the possibility that the limited effects of VEGF-targeted therapies and emergence of resistance might be due, in part, to VEGFA effects on ovarian CSC has not been evaluated. To investigate whether VEGFA stimulates ovarian CSCs, we used three models. Since > 60% of OVCAs express the estrogen receptor α (ER), we used the well-established ERα line, PEO1R, derived from human high-grade serous OVCA ascites (Langdon et al., 1994). Results were validated using an ER- high-grade serous human OVCA8 line (Slayton, 1984; Domcke et al., 2013). Since OVCA lines diverge from primary tumors over time (Ince et al., 2015) and since OVCA may represent different cancers with different molecular origins, results were validated using early-passage OCI-CSX, a primary OVCA culture derived from a clear cell OVCA. OCI-CSX faithfully represents the molecular and cellular phenotype of the original patient’s clear cell tumor and is one of 25 new ovarian cancer cultures established by Ince by immediate culture of primary cancer in Ovarian Carcinoma Modified Ince medium, OCMI (Ince et al., 2015).

Sphere formation from a single CSC seeded in low adhesion conditions is a measure of stem-like cell abundance in vivo (Visvader & Lindeman, 2012). Prior work showed VEGFA and a network of pro-inflammatory cytokines increase breast CSC abundance, but required prolonged exposure for full effect (Zhao et al., 2014; Picon-Ruiz et al., 2016). VEGFA (10 ng/ml) effects were assayed over short- and long-term exposures (1, 3, and 7 days). A 7-day exposure, but not shorter intervals, significantly increased sphere formation by PEO1R, OVCA8, and OCI-CSX cells seeded without further VEGFA. All sphere assays were carried out in limiting dilutions and sphere formation could not be accounted for by aggregation. VEGFA-blocking antibody, bevacizumab, and 2C3 antibody that blocks VEGFR2 both inhibited VEGFA-stimulated sphere formation (Fig 1A). Neither antibody alone decreased baseline sphere formation, suggesting VEGFA does not drive basal CSC self-renewal, but augments CSC recruitment in these OVCA models. VEGFA was not a mitogen in these models. Cell cycle profiles were not changed by VEGFA exposure for 48 h in 2D and were not affected by 7 days in sphere culture (representative data from OVCA8, Fig EV1A). Furthermore, the % apoptotic cells in PEO1R as assayed by annexin V staining was unchanged by VEGFA exposure for 48 h in 2D and were not affected by 7 days in sphere culture (representative data from OVCA8, Fig EV1A). Furthermore, the % apoptotic cells in PEO1R as assayed by annexin V staining was unchanged by VEGFA exposure for 48 h in 2D and were not affected by 7 days in sphere culture (representative data from OVCA8, Fig EV1A). Furthermore, the % apoptotic cells in PEO1R as assayed by annexin V staining was unchanged by VEGFA exposure for 48 h in 2D and were not affected by 7 days in sphere culture (representative data from OVCA8, Fig EV1A). Furthermore, the % apoptotic cells in PEO1R as assayed by annexin V staining was unchanged by VEGFA exposure for 48 h in 2D and were not affected by 7 days in sphere culture (representative data from OVCA8, Fig EV1A).

The ALDH1+ population of OVCA lines (Silva et al., 2011; Shah & Landen, 2014) and primary tumors (Stewart et al., 2011) is enriched for CSC in vitro and tumor-initiating cells in vivo. VEGFA exposure increased ALDH1+ cell abundance in all three models, including early-passage OCI-CSX culture (Ince et al., 2015; Fig 1B). Thus, prolonged VEGFA exposure increases the abundance of sphere forming and ALDH1+.

VEGFA increases expression of the stem-like cell regulator Bmi1

Several embryonic stem cell transcription factors (ES-TFs) govern embryonic stem cells (ES) self-renewal, induce pluripotency in skin fibroblasts (Li et al., 2010), and have been implicated in CSC self-renewal in a number of different cancers (Zhang et al., 2008). B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) is part of the polycomb-repressive complex 1 (PRC1) that regulates chromatin remodeling during development (Siddique & Saleem, 2012). Bmi1 promotes normal hematopoietic and neural stem cell expansion (Park et al., 2003; Molofsky et al., 2005) and can upregulate malignant stem-like cells in part through changes in ES-TFs (Lessard & Sauvageau, 2003; Siddique & Saleem, 2012). Bmi1 has
been shown to be upregulated in cancers, including OVCA (Siddique & Saleem, 2012), but its role in ovarian CSC expansion has not been established.

To assay their potential involvement in VEGFA-stimulated ALDH1 activity and sphere formation, we tested VEGFA effects on Bmi1 and ES-TFs in PEO1R, OVCAR8, and OCI-C5X. Basal Bmi1 levels were increased by 6 h and remained elevated at 7 days (Fig 2A, left). Densitometry of Western blots on repeat assays showed Bmi1 protein increased by 2.0 ± 0.02 fold in PEO1R, 4.7 ± 0.1 fold in OVCAR8, and 1.9 ± 0.09 fold in OCI-C5X after 7 days of VEGF exposure. Notably, while VEGFA also increased ES-TF levels, including cMyc (by 3.5 ± 0.1 fold by day 1), Oct4 (2.2 ± 0.06 fold, day 2), and Klf4 (4.1 ± 0.3 fold day 2; Fig EV2), these rose after the increase in Bmi1. Thus, we assayed the role of Bmi1 in VEGFA-mediated ovarian CSC effects.

**Increased sphere formation after VEGFA exposure is Src- and Bmi1-dependent**

Src is frequently overexpressed in human OVCA (Simpkins et al, 2012) and promotes tumor growth (Kim et al, 2009). Since Src was recently shown to mediate cytokine-driven CSC upregulation (Picon-Ruiz et al, 2016), we tested its potential as a mediator of effects of VEGFA on stem-like cells. VEGFA caused sustained Src activation with an increase in pSrc of 4.1 ± 0.42 fold in PEO1R, and 4.8 ± 0.08 fold in OVCAR8 and 1.8 ± 0.04 fold in OCI-C5X within...
VEGFA relieves miR-128 targeting of the regulated miR-128 in all OVCA models (Fig 4A). To test whether frequent work used primers to detect mature miR-128. VEGFA downregulated miR-128-2 pre-miR-128-1 to cells and 1/20,313 in VEGFA-exposed cells pre-treated with siRNA cells was 1/2,018, compared with 1/21,607 in non-VEGFA-exposed (Fig EV3). The tumor-initiating cell frequency in VEGFA-exposed et al (Fig EV1).

Effects of VEGFA and Bmi1 on ovarian tumor-initiating cell abundance were further investigated in vivo. Limiting dilution tumor-initiating cell assays, injecting between 100 and 100,000 cells, showed that sustained VEGFA exposure over 7 days prior to injection increased PEO1R tumor-initiating cell abundance. Ex vivo exposure to VEGFA decreased tumor latency and more animals formed tumors from VEGFA-exposed cells than from cells without VEGFA pre-treatment. Bmi1 knockdown prevented the VEGFA-mediated increase in tumor-initiating cell abundance (Fig 3A). Note that VEGFA was not a mitogen in this model and did not affect apoptosis (Fig EV1). Bmi1 siRNA did not impair proliferation or viability (Fig EV3). The tumor-initiating cell frequency in VEGFA-exposed cells was 1/2,018, compared with 1/21,607 in non-VEGFA-exposed cells and 1/20,313 in VEGFA-exposed cells pre-treated with siRNA to Bmi1, as calculated by L-Calc™ Limiting Dilution Software (Fig 3B). Thus, VEGFA increases tumor-initiating OVCA cell abundance in vivo and this is Bmi1 dependent.

VEGFA repression of miR-128-2 is Src dependent

Bmi1 is regulated by miR-128, a 21 nucleotide (ucacaguaaccgguacuçu) that targets the Bmi1 3’ UTR (Godlewski et al, 2008; Zhu et al, 2011; Jin et al, 2014). Mature miR-128 is encoded by two miRs, miR-128-1 and miR-128-2. qPCR with primers that distinguish pre-miR-128-1 and pre-miR-128-2 showed that VEGFA significantly reduced miR-128-2 but not miR-128-1 expression after 7 days (Fig EV4). Since pre-miRs are unstable and less abundant, subsequent work used primers to detect mature miR-128. VEGFA downregulated miR-128 in all OVCA models (Fig 4A). To test whether VEGFA releives miR-128 targeting of the Bmi1 3’ UTR, VEGFA-exposed and control cells were transfected with a Bmi1 3’ UTR luciferase reporter. VEGFA increased Bmi1 3’ UTR luciferase reporter activity in both OVCA lines and in OCI-C5X (Fig 4B). Thus, Bmi1 upregulation by VEGFA results from decreased inhibitory occupancy of the Bmi1 3’ UTR.

To test whether Src activation drives VEGFA-mediated miR-128 loss, cells were treated with or without VEGFA, and with or without saracatinib (AZD0530). Saracatinib increased basal miR-128 and abrogated miR-128 downregulation by VEGFA in all three models (Fig 4C). Effects of the Src family kinase inhibitor, saracatinib, were verified using siRNA-mediated SRC knockdown. Since saracatinib effectively inhibits several Src kinase family members, the requirement for Src was verified using SRC knockdown by siRNA with three different oligonucleotides. SRC knockdown increased basal miR-128 expression, and SRC siRNA transfection 48 h prior to addition of VEGFA abrogated the VEGFA-driven loss of miR-128 expression in both OVCA lines and in the primary OCI-C5X culture (representative data, Fig 4D). SRC knockdown not only increased basal miR-128, but it also prevented the VEGFA-driven increase in sphere formation. AntagomiR-128 rescued the inhibition of sphere formation by siSRC (Fig 4E), consistent with the notion that VEGFA-mediated action on miR-128 is downstream of Src. Thus, Src appears to govern basal miR-128 expression and Src activation is required for miR-128 repression following VEGFA exposure.

VEGFA repression of miR-128 requires DNA methyltransferase activity

VEGFA exposure had effects long after VEGFA withdrawal to increase OVCA tumor initiation in vivo (Fig 3); thus, we speculated that VEGFA might regulate miR-128-2 epigenetically. The extent to which miRNAs are epigenetically regulated is not fully understood (Suzuki et al, 2012). Tumor suppressor miRs are downregulated in cancers (Croce, 2009) through DNA methylation, since their repression is relieved by the methylation inhibitor 5’-azacytidine (Saito et al, 2006; Suzuki et al, 2012). While such miRNAs are methylation sensitive, the methyltransferases governing miRNA expression have not been fully characterized. 5’-azacytidine increased miR-128 expression in controls and blocked miR-128 downregulation by VEGFA in PEO1R and OCI-C5X (Fig 4F). Thus, VEGFA downregulation of miR-128 requires DNA methyltransferase action.

VEGFA upregulates Bmi1 through Src-mediated DNMT3A induction and miR-128-2 methylation

DNA methyltransferase (DNMT) 3A plays an important role to restrain gene expression and is deregulated in human malignancies (Fernandez et al, 2012). To test potential DNMT involvement in VEGFA-driven miR-128-2 repression, and since DNA methyltransferases, DNMT3A, DNMT3B, DNMT1, are often coregulated and elevated in cancer stem-like cells (Yang et al, 2015), we assayed all three methyltransferases. VEGFA increased DNMT3A but not DNMT1 and DNMT3B expression (Fig EV5A). Src inhibition decreased DNMT3A expression in untreated cells and prevented the increase in DNMT3A expression by VEGFA (Fig 5A, left). DNMT3A protein was increased by 2.6 ± 0.11 fold in PEO1R and by 1.6 ± 0.08 fold in OCI-C5X after 7-day VEGFA exposure (Fig 5A and B). Addition of saracatinib (AZD0530) abolished the VEGFA-stimulated DNMT3A increase and decreased basal DNMT3A protein levels (Fig 5A, right). Notably, siRNA-mediated SRC knockdown decreased basal DNMT3A and Bmi1 levels and prevented the VEGFA-driven upregulation of both DNMT3A and Bmi1 in PEO1R and OCI-C5X (Fig 5B).

DNMT3A knockdown modestly decreased baseline sphere formation and prevented the increase in sphere formation by
VEGFA (Fig 5C). DNMT3A was required for both VEGFA-driven loss of miR-128 and for the increase in Bmi1 protein (Fig 5D and E). Thus, VEGFA-driven miR-128 repression, Bmi1 upregulation, and stem-like cell expansion appear to be DNMT3A dependent.

miRNA-128-2 DNA methylation was analyzed by genomic bisulfite sequencing in PEO1R and in early-passage OCI-C5X. The miR-128-2 region contains ten CpG sites in a 347-bp sequence (nucleotide 166 to +181; Fig 5F). VEGFA increased the frequency of methylated CpGs in miR-128-2 in both PEO1R and OCI-C5X (Figs 5G and EV5B) consistent with its downregulation of miR-128 expression.

Saracatinib (AZD0530) and 5’-azacytidine both decreased baseline methylation and prevented the VEGFA-mediated increase in methylated CpGs in miR-128-2 (Figs 5G and EV5B). Notably, baseline miR-128 expression was lower and miR-128-2 methylation was higher in PEO1R than in early-passage OCI-C5X (Figs 5G and EV5B and C), supporting the notion that miR-128-2 expression is governed by DNA methylation. DNA methylation of miR-128-2 was strongly inversely correlated with its expression in both PEO1R (R² = –0.7513) and in the primary OCI-C5X models (R² = –0.9559; Fig 5H).

A more detailed kinetic time course showed that short-term VEGFA exposure rapidly activates VEGFR2pY1175 leading to

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**Figure 2.** VEGFA increases OVCA sphere formation via Src-mediated Bmi1 upregulation. VEGFA effects on indicated proteins.
See also Figs EV2 and EV3. 
A, B Bmi1 (A), and total and Y416-phosphorylated Src (pSrc) (B), at times indicated.
C Cells were treated for 7 days with or without VEGFA, ± Src inhibition by 1 μM saracatinib (AZD0530) during the last 48 h. Western blots show Src, pSrc, and Bmi1 levels.
D Cells were transduced with siRNA Bmi1 or scrambled controls 48 h prior to VEGFA treatment for 7 days and then recovered for Western blot.
E Cells were transduced with either siBmi1 or control siRNA for 48 h prior to VEGFA addition for 7 days (± siBmi1) or treated with VEGFA for 7 days with or without Src inhibitor (AZD0530, 1 μM) during the last 48 h followed by a 2-day washout without drug or cytokine prior to plating of spheres into limiting dilution sphere formation. All graphed data show mean ± SEM for at least 3 different biologic experiments with at least three technical repeats within each assay. Differences between multiple treatment groups were compared by ANOVA. PEO1R, 1,000 cells: *P < 0.0001, **P < 0.0001, †P = 0.0003, ‡P = 0.0014, OVCAR8: 2,500 cells: *P < 0.0001, **P = 0.0028, †P = 0.0297, ‡P = 0.6099, #P = 0.5492, 1,250 cells: *P = 0.0001, **P = 0.9344, †P = 0.9889, ‡P = 0.8895, #P = 0.5744, OCI-C5X: 4,000 cells: *P < 0.0001, **P = 0.0381, †P = 0.0381, ‡P = 0.2128, ‡P = 0.0545, 2,000 cells: *P = 0.0008, **P = 0.9399, †P = 0.6718, ‡P = 0.2289, ‡P = 0.3716.
increased SrcpY416. Both DNMT3A and Bmi1 increased gradually within the first 6–12 h after VEGFA addition. miR-128 levels, in turn, fell significantly within the first 6–12 h, and the decline was sustained and progressive over the next 7 days (Fig 6A and B). Thus, while VEGFR2 and Src signaling is triggered within minutes, the signal increases progressively and the epigenetic consequences of Src-dependent DNMT3A upregulation and miR-128 loss were manifest progressively over the successive 3–4 DNA replication cycles over 7 days. Notably VEGFA gene expression was also increased over the prolonged time course, providing a feed-forward mechanism to further activate Src and maintain this process (Fig 6C). In keeping with an epigenetic effect and not merely a signaling-dependent mechanism, the increase in sphere-forming ability was not fully felt until after several cell divisions: VEGFA exposure for 48 h did not significantly increase sphere; a 7-day exposure was required (Fig 6D). These data support that long-term VEGFA exposure is required to increase the abundance of CSC.

**VEGFA confers self-renewal of ALDH1+ cells**

Our initial assays showed VEGFA increased the proportion of ALDH1+ cells. We next assayed Src activation, DNMT3A levels, and Bmi1 in cells with different ALDH1 activity. When ALDH1-positive and ALDH1-negative populations were isolated by flow cytometry, ALDH1+ PEO1R cells showed higher Bmi1 (5.1 ± 0.08 fold), Src activation (pSrc, 1.8 ± 0.02 fold), and DNMT3A levels (1.5 ± 0.07 fold) than ALDH1- cells, supporting the notion that Src and Bmi1 are drivers of ovarian CSCs in this model (Fig 7A). Furthermore, ALDH1+ population also expressed lower miR-128 than ALDH1- cells (Fig 7B).
miR-128
VEGFA + AZD0530
VEGFA
AZD0530
VEGFA
VEGFA
VEGFA
Ctrl
310
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Figure 4. VEGFA downregulates miR-128 expression in a Src-dependent manner.
A VEGFA treatment for 7 days decreases miR-128 expression as assayed by qPCR. *P = 0.03, **P = 0.0000003, †P = 0.01.
B VEGFA increases BMI 3' UTR luciferase activity. *P = 0.00015, **P = 0.00046, †P = 0.0022.
C Effects of VEGFA alone, of saracatinib (AZD0530, 1 μM) alone, or saracatinib added during the final 48 h of 7-day VEGFA treatment on miR-128 expression compared with mock-treated controls (Ctrl). PEO1R, *P = 0.0005, **P = 0.00005, †P = 0.5522, OVCAR8, *P = 0.0359, **P < 0.0001, †P < 0.0001, OCI-C5X, *P = 0.0064, **P = 0.0123, †P = 0.037.
D Effects of VEGFA alone for 7 days, of siRNA to SRC (siSRC) alone, or siSRC transfection 48 h prior to addition VEGFA for 7 days on miR-128 expression compared with mock-treated controls (Ctrl). PEO1R, *P = 0.019, **P = 0.0008, †P = 0.047, OCI-C5X, *P = 0.027, **P = 0.016, †P = 0.024.
E Cells were transduced with either siSRC or control siRNA for 48 h prior to VEGFA addition for 7 days (± siSRC), or treated with antagomiR-128 for 48 h with or without siSRC and plated into sphere assays. *P = 0.0026, **P = 0.0052, †P = 0.076, ‡P = 0.0014, †P = 0.014. AntagomiR-128 decreases miR-128 expression as assayed by qPCR. *P = 0.012.
F Effects of 7 days of VEGFA ± 1 μM 5’-azacytidine (5’aza) on miR-128 expression in lines indicated. PEO1R, *P = 0.0451, **P < 0.0001, †P < 0.0001, OCI-C5X, *P = 0.0048, **P = 0.0003, †P = 0.0033.

Data information: All graphed data show mean ± SEM for at least 3 different biologic experiments with at least three technical repeats within each assay. Differences between two groups were assayed by Student’s t-test, and multiple treatment groups were compared by ANOVA. See also Fig EV4.

The ALDH1⁺ population contained more sphere-forming cells than ALDH1⁻ cells (Fig 7C). VEGFA treatment of sorted populations for 7 days significantly increased the % of sphere-forming cells only in the ALDH1⁺ (P = 0.0072), not the ALDH1⁻ subpopulation (P = 0.21; Fig 7C). Thus, prolonged VEGFA exposure increases the abundance of sphere-forming cells, but this effect is limited to an ALDH1⁺ target population.

To further investigate VEGFA effects on ovarian CSCs, ALDH1⁺-positive and ALDH1⁻-negative cells were isolated by flow cytometry and grown with or without VEGFA over 8 days. The number and phenotype of their progeny were assayed every 2 days. The progeny of both ALDH1⁺ and ALDH1⁻ cells increased at similar rates in 2D culture (Fig EV6A). The growth of either ALDH1⁺ or ALDH1⁻ populations over the next 8 days was similar with or
Figure 5. VEGFA-driven sphere formation and Bmi1 upregulation are Src and DNMT3A dependent and mediated by DNA methylation of miR-128-2.

A Effects of 7 days of VEGFA on DNMT3A expression by qPCR (left) or Western blot (right) ± Src inhibition (1 μM AZD0530) over the final 48 h in PEO1R. *P = 0.0031, **P = 0.0051, †P = 0.0163.

B–E (B) PEO1R or OCI-C5X cells were transduced with siRNA to SRC or scrambled controls 48 h prior to VEGFA treatment for 7 days. Western blots show Src, pSrc, Bmi1, and DNMT3A levels. (C–E) Cells were transduced with either siRNA to DNMT3A or control siRNA (± siDNMT3A) for 48 h prior to 7-day treatment ± VEGFA and then (C) sphere formation was assayed for PEO1R (1,000 cells) and OCI-C5X (4,000 cells) cells (PEO1R; *P = 0.0002, **P = 0.0061, †P = 0.0091, OCI-C5X; *P = 0.0013, **P = 0.0283, †P = 0.0001) and (D) effects of siDNMT3A on VEGFA-mediated miR-128 repression were assayed by qPCR (PEO1R; *P = 0.0149, **P < 0.0001, †P = 0.0023, OCI-C5X; *P = 0.029, **P < 0.0001, †P = 0.0005), and (E) DNMT3A and Bmi1 levels were assayed by Western blot.

F, G (F) miR-128-2 genomic region with number showing the position of Cpg sites from the 5′ end of pre-miR-128-2 that were tested by bisulfite sequencing. (G) Results of bisulfite sequencing of the miR-128-2 region in PEO1R. Open circles indicate unmethylated, and filled circles indicate methylated Cpg sites.

H Expression of miR-128 in PEO1R and OCI-C5X is inversely correlated with miR-128 methylation.

Data information: All graphed data show mean ± SEM for at least 3 different biologic experiments with at least three technical repeats within each assay. Differences between multiple treatment groups were compared by ANOVA. See also Fig EVS.
without VEGFA exposure (Fig EV6B and C). Untreated ALDH1+ cells gave rise to ALDH1-negative cells over time, compatible with asymmetric cell division (Fig 7D). VEGFA exposure increased the number of ALDH1+ cells generated from ALDH1+ cells compared with vehicle-treated controls. The fraction of ALDH1+ cells remained higher and VEGFA decreased the generation of ALDH1+ from ALDH1+ precursors (Fig 7D). That VEGFA-treated ALDH1+ cells produced more ALDH1+ progeny over time (Fig 7E), is compatible with a VEGFA-induced shift from asymmetric division toward self-renewal of ALDH1+ ovarian CSC. Notably, sorted ALDH1+ cells yielded only ALDH1- progeny over time (Fig EV6D). Taken together, our data are consistent with baseline activation of Src governing DNMT3A expression and Bmi1 in the ALDH1+ population. This pathway appears to be further activated following VEGFA stimulation.

High VEGFA and decreased miR-128-2 associate with poor OVCA outcome

High intratumor VEGFA correlates with poor outcome for various cancers, but OVCA studies have been limited by small numbers and not all show significance (Yu et al, 2013). A recent OVCA meta-analysis indicated high intratumor VEGFA associates with poor outcome, but reported heterogeneity among studies and a greater prognostic import for VEGFA in early- than in late-stage disease (Yu et al, 2013). We evaluated VEGFA gene expression by Kaplan-Meier analysis in over one thousand primary OVCA (n = 1,305 for progression-free survival, PFS) using http://kmplot.com/analysis. OVCA with reduced VEGFA expression had significantly better PFS, shown for the lowest quartile of VEGFA [HR 1.37, 95% CI (1.18–1.6), P = 3.9e-05] and below the median [HR 1.23, 95% CI (1.08–1.41), P = 0.0019] (Fig 8A).

Among four hundred and eighty five primary high-grade serous ovarian cancers (HGSOCs) in The Cancer Genome Atlas (TCGA) database with gene and miRNA expression, and outcome data available, tumors with the lowest miR-128-2 expression associated with the worst disease-free survival (DFS, n = 396, P = 0.038) and overall survival (OS, n = 485, P = 0.023; Fig 8B). HGSOC in the top VEGFA expression quartile with the lowest quartile of miR-128-2 expression showed a significantly worse disease-free survival (DFS) than those with high miR-128-2 levels [DFS 14.8 versus 21.6 months; HR 1.98, 95% CI (1.04–3.75), P = 0.034] (Fig 8C), supporting the notion that a subset of aggressive OVCA have high VEGFA-driven loss of miR-128-2 expression.

Figure 6. Kinetics of VEGFA-mediated VEGFR2 and Src activation, DNMT3A upregulation, miR-128 decline, and increased Bmi1 and sphere formation.

A VEGFA effects on indicated proteins in PEO1R cells at indicated times in minutes or hours.
B VEGFA effects on miR-128 in PEO1R cells at indicated times in hours (h) or days (D). *P = 0.0379, **P = 0.2627, †P = 0.0424, ‡P = 0.0073, ††P = 0.0001.
C VEGFA effects on VEGFA in PEO1R cells at indicated times. *P = 0.1124, **P = 0.003.
D Indicated cells were pre-treated in 2-day culture for 2 or 7 days with 10 ng/ml VEGFA or vehicle only and then plated into sphere assays. *P = 0.2073, **P = 0.0006.

Data information: All graphed data show mean ± SEM for at least 3 different biologic experiments with at least three technical repeats within each assay. Differences between multiple treatment groups were compared by ANOVA.
that Bmi1 regulates ES-TFs, including Sox2 and Klf4 (Molofsky et al., 2005; Siddique & Saleem, 2012).

Tumor suppressor miRs are frequently extinguished in cancers. For example, miR-148a, miR-34b/c, and miR-9 downregulate oncogenes, including c-MYC, E2F3, CDK6, and TGFβ2 and are often reduced in cancers. 5′-azacytidine restored miRNA expression, indicating that DNA methylation repressed these tumor suppressor miRNAs to increase metastasis (Suzuki et al., 2012). Methylation also downregulates miR-129-2 to induce SOX4 (Crocce, 2009), and miR-143 to upregulate the MLL-AF4 oncogene in leukemia (Dou et al., 2012). While many such miRNAs are methylation sensitive, the specific methyltransferases governing their expression have not been identified. Present work links a cytokine, VEGFA, with Src pathway activation and identifies DNMT3A as a novel regulator of site-specific mir-128-2 methylation, Bmi1 upregulation, and expansion of stem-like OVCA cells. Our data are consistent with recent findings that DNA methyltransferase inhibition by SGI-110 reduced the abundance of OVCA cells with stem-like properties including ALDH1+ in vitro and tumor-initiating cells in vivo, and resensitized cisplatinum-resistant A2780 cells to platinum therapy (Wang et al., 2014). In both the PEO1R line and primary OCI-C5X culture, miR-128 expression was strongly inversely correlated with miR-128-2
methylation ($R^2 = -0.7513$ and $R^2 = -0.9559$, respectively). Interestingly, the early-passage primary OVCA culture, OCI-C5X, showed considerably less miR-128-2 methylation and higher miR-128-2 expression than the extensively passaged, highly tumorigenic PEO1R line, potentially reflecting greater malignancy of the original PEO1R tumor in vivo or progression during culture.

Present data suggest that in contrast to hematologic malignancies where inactivating DNMT3A mutations reveal a tumor suppressor role (Fernandez et al., 2012; Yang et al., 2015), DNMT3A plays an oncogenic role as a key effector of VEGFA-driven ovarian CSC expansion. Epigenetic regulators, including EZH2 and DNA methyltransferases, appear to have dual roles, acting as tumor suppressors or oncogenes in different malignancies, depending on the differentiation state of cells undergoing malignant change (Fernandez et al., 2012). Present work suggests that DNMT3A also plays a dual role. In addition to its tumor suppressor role in leukemia, DNMT3A also mediates stem-like cell expansion in ovarian cancers. VEGFA induced DNMT3A, but not related DNMT3B or DNMT1, and DNMT3A knockdown prevented VEGFA-mediated miR-128 loss and the increases in Bmi1 expression and OVCA tumor sphere formation in vitro. BMI1 knockdown abolished VEGFA-dependent increases in sphere formation and in ovarian tumor-initiating cells in vivo. DNMT3A appears to act downstream of Src, which is activated in over 70% of OVCA

![Figure 8](image-url)

**Figure 8.** Prognostic value of VEGFA and miR-128-2 expression in ovarian cancer patients.

A Kaplan–Meier plot of progression-free survival (PFS) of OVCA patients, stratified by VEGFA expression of using KM Plotter (http://kmplot.com/analysis/). Patients with available clinical data: PFS, $n = 1,305$.

B Kaplan–Meier plot of disease-free survival (DFS) and overall survival (OS) of TCGA high-grade serous ovarian cancer (HGSOC) patients, stratified by miR-128-2 expression using cBioPortal (http://www.cbioportal.org/). Patients with available clinical data: DFS, $n = 396$; and OS, $n = 485$.

C Workflow for TCGA data classification. Clinical data for HGSOC DFS were obtained from cBioportal, which are graphed by VEGFA and miR-128-2 levels.
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Flow sorting of ALDH1⁺ or ALDH1⁻ cells

Cells were stained with ALDEFLUOR™ (Stem Cell Technologies, Durham, NC) and then ALDH1⁺ or ALDH1⁻ cells separated by FACSAria II (BD Biosciences). Lineage tracing with MitoTracker dyes (Thermo Fisher Scientific and Invitrogen) was not possible because these dyes all caused cytotoxicity.

Tumor-initiating stem-like cell assays

The University of Miami (UM) Animal Care and Use Committee approved all animal work. For limiting dilution xenograft assays, PEO1R was transduced or not with siBMI1 or scrambled siRNA (Santa Cruz Biotech) controls for 48 h and then treated with ± VEGFA 10 ng/ml for 7 days. One hundred and twenty-four 5-week-old, female NOD/SCID mice were injected with cells from four experimental groups (untreated scrambled siRNA controls, VEGFA-treated scrambled siRNA controls, VEGFA-treated siBMI1-transduced, and siBMI1-transduced). Animals were injected in the mammary fat pad with 100 (10 mice/group), 1,000 (eight mice/group), 10,000 (eight mice/group), or 100,000 (five mice/group) cells in 100 µl Matrigel (BD Biosciences) as in Ginestier et al (2007). Tumors were measured twice per week. Mice without tumors were followed at least 9 months as per UM Animal Care and Use Committee standards.

miRNA RT–PCR, siRNA, antagoniR-128, and Western blot analysis

miRNA isolation was carried out using miRNeasy (Qiagen) in cells treated with or without prior VEGFA, saracatinib, or 5′-azacytidine (Sigma-Aldrich). cDNA synthesis used Ncode miR First Strand cDNA synthesis kit (Invitrogen). qPCR of miR-128 used mir-128 forward: 5′-TGTGTTAAGGTTAGGGAATTAAATTAG, reverse: TCAACA AAAATACACAACTCTTC (Zhao et al, 2012; Mihaly et al, 2013). The KM curves for disease-free survival and OS with miR-128-2 expression used TM plotter (http://kmplot.com/analysis/) to create Kaplan–Meier (KM) curves for PFS and OS versus VEGFA expression (Gyorffy et al, 2012; Mihaly et al, 2013). The KM curves for disease-free survival and OS with miR-128-2 expression used TM plotter. OVCAs with high VEGFA and high or low miR-128-2 expression from TCGA were compared by cBioPortal to create Kaplan–Meier curves.

Statistical analysis

For in vitro work, data are graphed from ≥ 3 biologic experiments as means ± standard error of the mean (SEM). Comparisons of > two groups were made using one-way analysis of variance (ANOVA) followed by Student–Newman–Keul’s post hoc analysis or two-way ANOVA followed by Bonferroni post hoc tests. Comparisons of two groups was made using Student’s t-test. Tumor-initiating cell frequency was calculated by L-Calc Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies.

Study approval

All animal work was UM SOM IACUC-approved. All analyses of human OVCA for VEGFA or miRNA-128 were based on in silico analysis of retrospective datasets including National Center for Biotechnology Information and the OVCA TCGA dataset using cBioPortal. This work is not considered “Human Subjects Research”.

Expanded View for this article is available online.

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Author contributions

KJ and MK designed and performed experiments, analyzed data, and wrote the paper. CC and FS designed and/or performed experiments and analyzed data. KJ and MK performed statistical data analysis. TAI provided primary ovarian cancer cultures and methodology for their culture and assisted with experimental design. JMS designed experiments, obtained funding to support the work, analyzed data, and wrote the paper.
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The authors declare that they have no conflict of interest.

References


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