Pericytes Promote Malignant Ovarian Cancer Progression in Mice and Predict Poor Prognosis in Serous Ovarian Cancer Patients

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Abstract

Purpose: The aim of this study was to investigate the role of pericytes in regulating malignant ovarian cancer progression.

Experimental Design: The pericyte mRNA signature was used to interrogate ovarian cancer patient datasets to determine its prognostic value for recurrence and mortality. Xenograft models of ovarian cancer were used to determine if co-injection with pericytes affected tumor growth rate and metastasis, whereas coculture models were utilized to investigate the direct effect of pericytes on ovarian cancer cells. Pericyte markers were used to stain patient tissue samples to ascertain their use in prognosis.

Results: Interrogation of two serous ovarian cancer patient datasets [the Australian Ovarian Cancer Study, \(n = 215\); and the NCI TCGA (The Cancer Genome Atlas), \(n = 408\)] showed that a high pericyte score is highly predictive for poor patient prognosis. Co-injection of ovarian cancer (OVCAR-5 & -8) cells with pericytes in a xenograft model resulted in accelerated ovarian tumor growth, and aggressive metastases, without altering tumor vasculature. Pericyte co-culture in vitro promoted ovarian cancer cell proliferation and invasion. High \(\alpha\)SMA protein levels in patient tissue microarrays were correlated with more aggressive disease and earlier recurrence.

Conclusions: High pericyte score provides the best means to date of identifying patients with ovarian cancer at high risk of rapid relapse and mortality (mean progression-free survival time <9 months). The stroma contains rare yet extremely potent locally resident mesenchymal stem cells—a subset of “cancer-associated fibroblasts” that promote aggressive tumor growth and metastatic dissemination, underlying the prognostic capacity of a high pericyte score to strongly predict earlier relapse and mortality. Clin Cancer Res; 22(7): 1813–24. ©2015 AACR.

Introduction

Ovarian cancer is the most life-threatening gynecologic cancer, with very high rates of recurrence and mortality following diagnosis. Statistics from the United States (NCI) and United Kingdom (CRUK)—countries with some of the highest rates of ovarian cancer—show that although diagnosis at stage 1 at a young age is associated with approximately 90% survival, the overwhelming majority of patients with ovarian cancer (~85%) are diagnosed at advanced stages of disease [3 and 4], after metastatic dissemination.
equally plausible that the cooperative stroma is essential for malignant progression, it is the prevailing view that the TME is pro-tumorigenic. Although sufficient evidence exists to support the notion that recruitment of a cooperative stroma is essential for malignant progression, it is equally plausible that the influx of some stromal elements is the body’s attempt to limit cancer spread through fibroblastic encapsulation typical of many tumor types. Notably, the TME has been implicated in contributing at least partially to resistance against cancer therapeutic reagents (10), but also in enhancing therapeutic efficacy depending on context [see ref. (11) for review].

It is, therefore, vital that the functional heterogeneity and diverse origins of the tumor stroma are more fully mapped. It is well known that CAFs isolated from cancers promote tumor growth, invasiveness, and metastasis of many cancers, notably breast, prostate, and pancreatic carcinomas (12–14) compared with normal fibroblasts. However, the postulated origins of CAFs include many normal stromal cells, including tissue resident myofibroblasts, activated adipocytes, and BM-MSCs (13, 15)—the latter representing perhaps the best defined source of CAFs. However, BM-MSCs that are home to developing tumors inducing increased metastases comprise approximately 20% of CAFs (16–20), leaving some 50% to 80% of CAFs that are not BM-derived and may arise from locally resident fibroblasts and presumably MSC-like populations such as pericytes that form the focus of this study.

Pericytes are best known for regulating endothelial cell proliferation, differentiation, and microvascular perfusion/permeability through paracrine regulators such as TGF-β and vasoactive agents (21, 22), and are identified as α-smooth muscle actin (α-SMA)–positive, contractile cells located abluminally in microvessels. It is increasingly evident that pericytes and BM-MSCs share many phenotypic and functional attributes, including multilineage differentiation capacity, and may have a pro-proliferative role in organ growth, repair, and regeneration (23–25). Notably, we have previously demonstrated that pericytes promote normal epithelial cell proliferation and regeneration in the absence of angiogenesis, most likely through the secretion of the LAMA5 isoform of laminin (26). In the context of cancer, targeting both endothelial cells and pericytes through kinase inhibitors of VEGF, which promotes blood vessel growth, and PDGF-B, which promotes proliferation and survival of pericytes (27), improved the efficacy of anti-cancer therapies in animal models (28–31), attributed to destabilizing microvascular structure (27). Although subsequent studies claimed to show unaffected tumor growth following pericyte ablation, complete pericyte knockdown was not achieved by treatment with AX102—an inhibitor of PDGF-B signaling (32), or in PDGFB$	ext{ret/ret}$ mice harboring a mutation in the PDGF-B retention motif (33) with only partial decrease in tumor vasculature and pericyte number. A maximal 50% reduction in pericyte number is reached in PDGFB$	ext{ret/ret}$ mice (34). In contrast, comprehensive knockdown of pericytes via NG2-promoter–driven thymidine kinase results in tumor hypoxia, leading to epithelial–mesenchymal transition (EMT) and increased metastatic lung dissemination in mouse models of breast cancer, melanoma, and renal cell carcinoma (35). In fact, retaining pericytes within tumor blood vessel walls may limit metastatic spread through leaky blood vessels (36).

Thus, arguments to support both tumor growth–limiting and metastasis-promoting roles have been made for pericytes and most likely depend on the nature of the experimental model used. In this study, we show that the molecular signature of pericytes is highly predictive for patient relapse and mortality in high-grade serous ovarian cancer patients, demonstrating our ability to identify with a very high degree of certainty those patients who die in less than 9 months, despite aggressive treatment at diagnosis, suggesting a potent pro-tumorigenic/pro-metastatic role for pericytes in ovarian cancer progression. Consistent with this clinical correlate, we experimentally demonstrate that MSC-like pericytes are potent stimulators of both poorly and highly tumorigenic ovarian cancer cell lines when introduced into the TME in a xenograft model, accelerating tumor growth rates and earlier metastasis in aggressive ovarian cancer cells, but also inducing metastasis in nonmetastatic cell lines without affecting tumor vasculature. These influences in malignant ovarian cancer cell proliferation, migration, and invasion were also demonstrable in vitro using co-culture models, further indicating a novel and as yet unappreciated function for pericytes in malignant progression.

Materials and Methods

In silico analyses
Bioinformatics analyses for predicting prognosis based on the pericyte signature and gene set enrichment are described in the Supplementary data.

Cell culture
CD45 VLA-1+ pericytes and CD45 VLA-1− fibroblasts were isolated from human neonatal foreskin, as described (26) and expanded in culture up to passage 4 (p4) (24). Fibroblasts were maintained in DMEM with 10% FCS, and pericytes in EGM-2 (Lonza; #CC-4147). OVCAR-5 and OVCAR-8 cells obtained from NCI were authenticated using short tandem repeat markers to confirm cell identity against the Genome Project Database (Wellcome Trust Sanger Institute) and were maintained in RPMI 1640 (Invitrogen; #11875) with 10% FCS, 25 mmol/L HEPES buffer, 1% penicillin-streptomycin, and 1.5% DiFucan, and HEK293T cells in DMEM with 10% FCS, 1% L-glutamate (2 mmol/L), and 1% sodium pyruvate (1 mmol/L) without antibiotics for lentiviral production. OVCAR-5 cells and pericytes...
were transduced with GFP-luciferase, as described in the Supplementary data. All human tissue experimentation was approved by the Peter MacCallum Human Research Ethics Committee (#03/44).

### Animals

Six to eight week old female nude Balb/c mice (WEHI), housed in a pathogen-free 12-hour light–dark environment, fed *ad libitum* were used for tumorigenicity assays. All experimentation was approved by the Peter MacCallum Animal Research Ethics Committee (# E394).

### Tumorigenicity assay

OVCAR-5 cells (8 × 10^6) ± 10% fibroblasts or pericytes in 100 μL of 1:1 sterile PBS and standard Matrigel (BD Biosciences) were injected subcutaneously (s.c.) into the flanks of mice. Five mice were injected/group; all experiments performed in triplicate.

### Immunostaining and morphometric analyses, invasion assays

All procedures and antibodies used are described in the Supplementary data.

### Luciferase imaging

Metastatic spread and pericyte survival in *vivo* were monitored using the Xenogen Real-Time Imaging System. D-luciferin (Gold Biotechnology Inc.), a substrate for the luciferase enzyme, was injected s.c. at 150 μg/g body weight in PBS. Mice were allowed free movement for 6 to 8 minutes, anaesthetized with isoflurane, and imaged within 10 to 12 minutes of luciferin injection. Bioluminescent imaging (BLI) was repeated every 7 days to track metastatic movement for 6 to 8 minutes, anaesthetized with isoflurane, and imaged within 10 to 12 minutes of luciferin injection. Bioluminescent imaging (BLI) was repeated every 7 days to track metastatic spread until an experimental or ethical endpoint was reached, from d14 when primary tumors were palpable. At the endpoint, as the spread until an experimental or ethical endpoint was reached, from

### Vascular permeability assay

A volume of 100 μL of 10 mg/mL FITC-dextran (2,000,000 MW, Sigma) was injected into the tail vein an hour before sacrifice. Tumors were collected, snap-frozen in liquid nitrogen, and cryo-sections co-stained with the endothelial marker CD31 for fluorescence microscopy analysis.

### Tissue microarrays

Patient tissue microarrays (TMA) consisting of 4-μm cores of formalin-fixed, paraffin-embedded, high-grade serous ovarian cancer biopsy tissues were obtained from the Australian Ovarian Cancer Study (AOCS) approved by the AOCS review board.

### *In-vitro* proliferation assays

Co-culture proliferation assays were performed in a 6-well format. GFP* OVCAR-5 (2 × 10^4) cells alone or with 2 × 10^4 p4 pericytes or p4 fibroblasts were mixed and seeded in either 1% or 10% FBS epithelialization medium. Plates were incubated at 37°C, in 5% CO_2 for 24, 48, and 72 hours, fixed in 4% paraformaldehyde (w/v), and immunostained for GFP to determine the mass of OVCAR-5 cells over time.

### Statistical analysis

Data analyses were performed using the Prism 6.0 (Graphpad software) or R software. Pooled data were represented as mean ± SD, unless otherwise indicated.

### Results

The transcriptional profile of pericytes predicts significantly earlier relapse and mortality in high-grade serous ovarian cancer patients

The AOCS Group showed that high-grade serous ovarian cancer patients with a stromal signature had a poor clinical outcome (5), as reported for breast cancer patients (6). Previously we demonstrated that MSC-like pericytes had high potency in increasing epithelial proliferative capacity compared with fibroblasts (26), so we used the molecular signature of these two distinct stromal cell types (26), to compare their predictive capacity for clinical outcome in the AOCS ovarian cancer patient dataset in *silico*, using the OVCAS ovarian cancer stromal signature as a reference (5). Notably, the pericyte-specific signature or high pericyte score was a potent predictor of rapid relapse and mortality (P = 0.00067; Kaplan–Meier plots Fig. 1A), identifying those patients with a mean progression-free survival (PFS) time of 9 months or less versus those with a low pericyte score (mean PFS time of 29 months) despite similar treatment, as compared with the AOCS ovarian cancer stromal signature (P = 0.001; ref. 5) and the normal fibroblast signature (P = 0.01).

Analysis of genes co-expressed by laser-capture micro-dissected ovarian CAFs in the AOCS study and normal pericytes revealed 146 genes, including well-known pericyte markers (PDGFRB, ACTA2, RGS5, CALD1, MCAM, and ANGPT1) (26), growth factors, adhesion ligands and receptors (FGF/FGFRs, Tenascin C, LAMA3, LAMA5, CSPG-4, the VLA-1, VLA-3 and VLA-7 integrins), BMPs, and *Notch* pathway signaling genes (Supplementary Table S1), linked to CAFs and ovarian cancer progression (4). Importantly, minimal overlap was detected with the Gene Ontology classification angiogenesis signature (GO Angiogenesis—GO:0001525) with our pericyte signature, given that pericytes stabilize tumor vasculature, with only two common genes (angiopoietin 1 and 2). Moreover, no overlap was present between the recently described angiogenic signatures (37), suggesting that the significantly earlier relapse observed in patients with a high pericyte score was unrelated to angiogenesis. An interrogation of the NCI TCGA (The Cancer Genome Atlas) ovarian cancer patient dataset further confirmed the ability of the pericyte signature to predict decreased survival in a group of 408 patients (Fig. 1B; P = 0.008), leading us to investigate whether pericytes could promote ovarian tumor growth experimentally without affecting angiogenesis.

### Pericytes accelerate ovarian tumor growth in *vivo*

OVCAR-5 cells derived from a serous ovarian cancer patient with metastatic disease before treatment with anti-cancer agents (38) were used as an ovarian cancer model. GFP-luciferase* OVCAR-5 cells resuspended in Matrigel were injected s.c. in nude mice, either alone (OVCAR-5) or with pericytes (OVCAR-5+P) or fibroblasts (OVCAR-5+F), at a 10:1 tumor:stromal cell ratio. Pericyte co-injection consistently led to accelerated tumor growth compared with OVCAR-5 or OVCAR-5+F (Fig. 1C; P < 0.0001; n = 5 independent experiments), and increased endpoint tumor volumes (and mass) at
day 35 (Fig. 1D; \( P < 0.0001 \)); OVCAR-5+P tumors reached 200 mm\(^3\) 4 to 5 days earlier. Furthermore, a dose-dependent effect was observed when the proportion of pericytes was increased from 10% to 50%, keeping the number of OVCAR-5 cells constant, with greater endpoint tumor volumes than OVCAR-5 controls in both the 10:1 and 1:1 pericyte co-injected groups (\( P < 0.05 \) and \( P < 0.01 \), respectively; Fig. 1E).

Dual staining for epithelial-specific EpCam and Ki67 (with anti-human specific antibodies) revealed a direct increase in OVCAR-5 cell proliferation, i.e., number of EpCam\(^+\)/Ki67\(^+\) cells compared with OVCAR-5-only controls and OVCAR-5+F tumors (\( P < 0.0001 \) and \( P < 0.01 \) respectively; Fig. 1F). No difference in apoptotic index was observed between experimental groups at day 11 or day 35 by staining for cleaved caspase-3 (CC3; Supplementary Fig. S1A–S1H), excluding decreased apoptosis in increasing tumor size. Moreover, in vitro experiments showed that pericyte co-cultured GFP\(^+\) OVCAR-5 cells displayed increased proliferation compared with controls within 72 hours in both 1% (\( P = 0.0349 \)) and 10% serum (\( P = 0.0328 \)), not seen in fibroblast co-cultures (Supplementary Fig. S2A–S2C).

Injected pericytes persist but do not proliferate or contribute to angiogenesis in OVCAR-5 tumors

BLI of xenografts generated with unlabeled OVCAR-5 cells and GFP-luciferase\(^–\)-tagged pericytes permitted pericyte-tracking in developing tumors. Whilst control animals (OVCAR-5 cells alone) gave no signal despite luciferin injection (Fig. 2A), pericytes persisted within co-injected tumors at all time points analyzed (Fig. 2B). Histological analyses revealed single GFP\(^+\) pericytes in OVCAR-5+P tumors, declining in numbers over time (Fig. 2C). Notably, GFP\(^+\) pericytes were Ki67-negative at all time points, indicating that they did not proliferate during tumorigenesis (Fig. 2C).

We next addressed whether co-injected pericytes accelerated tumor growth by increasing or stabilizing tumor vasculature. The area of CD31\(^+\) blood vessels in tumors remained unaltered both

![Figure 1](https://example.com/figure1.png)

**Figure 1.**
The pericyte-specific gene signature predicts poor prognosis in patients with ovarian cancer, and co-injection of OVCAR-5 ovarian cancer cells with pericytes accelerates tumor cell proliferation and tumor volume. Kaplan-Meier curves showing a significantly poorer PFS rate among 215 high-grade serous ovarian cancer patients with a high score of pericyte-specific genes in the AOC5 dataset (A) and the NCI TCGA patient dataset (B). C, nonlinear regression fit of tumor volumes against time generated from the injection of \( 8 \times 10^5 \) OVCAR-5 cells alone or co-injected at a ratio of 10:1 with pericytes (OVCAR-5+P) or fibroblasts (OVCAR-5+F). Data represented as mean tumor volume \( \pm SD \) of 26 mice per group from 5 independent experiments. Repeated measure data for each time point were compared using two-way ANOVA. D, quantification of endpoint tumor volume at day 35 represented as mean tumor volume \( \pm SEM \) of 5 independent experiments, calculated from data shown in A. E, quantification of endpoint tumor volume at day 35, demonstrating the dose effect of increasing the number of pericytes on OVCAR-5 tumor growth, i.e., injection of \( 5 \times 10^5 \) OVCAR-5 cells with or without pericytes at a ratio of 10:1 and 1:1. Data are shown as mean tumor volume \( \pm SEM \) of 10 mice per group from 2 independent experiments. Statistical analysis in E and F performed using one-way ANOVA. F, quantification of dual immunofluorescent staining with anti-human specific antibodies to the proliferation marker Ki67 and the epithelial marker EpCam of ovarian tumors generated by OVCAR-5 cells alone or co-injected with pericytes—OVCAR-5+P and fibroblasts, OVCAR-5+F showing a significant increase in the number of Ki67\(^+\)/EpCam\(^+\) tumor cells in the OVCAR-5+P group. Data are shown as mean \( \pm SEM \) from 3 tumors per group from 2 independent experiments. Statistical analyses performed by one-way ANOVA. \( \cdot \cdot \cdot \ P < 0.05 \), \( \cdot \cdot \cdot \cdot \cdot \cdot \ P < 0.001 \), \( \cdot \cdot \cdot \cdot \cdot \cdot \cdot \ P < 0.0001 \), ns, not significant.
in the center and in the edges of the OVCAR-5+P tumors compared with controls (Fig. 3A and B) at day 11 (Fig. 3C; \( P = 0.1879 \)), confirmed further by measuring microvessel density (MVD) (Fig. 3D; \( P = 0.8910 \)). The CD31+ blood vessel area (Fig. 3E; \( P = 0.2021 \)) and MVD remained unaffected at day 35 (Fig. 3F; \( P = 0.7790 \)).

Furthermore, we could not find any differences in the \( \alpha \text{SMA}^+ \) pericyte coverage index (MPI) of CD34+ microvessels between OVCAR-5+P and OVCAR-5 controls (Fig. 3G; \( P = 0.5321 \)), indicating that pericyte inclusion did not alter the structural stability of OVCAR-5 tumor vasculature.

Similar analyses of microvessels in clinical samples, i.e., TMAs of serous ovarian cancer patients, demonstrated that CD34+ expression in tumor microvessels did not correlate with time to relapse or survival (Fig. 3H and I), providing independent verification that poor prognosis predicted by the pericyte signature had minimal overlap with the angiogenic signature.

### Figure 2.

Co-injected human pericytes survive but do not proliferate in ovarian tumors in vivo. A and B, representative BLI images of mice with unlabeled OVCAR-5 tumors or OVCAR-5+GFP-luciferase tagged pericytes imaged at days 6–35. BLI imaging conducted in 3 mice/group/time point in 2 replicate experiments. C, dual immunofluorescent staining for GFP+ pericytes (green) and Ki67+ proliferating cells (red) in days 6–35 pericyte co-injected tumors, showing decline in pericyte numbers over time and absence of Ki67+ GFP+ pericytes. Images are representative of three random fields from 3 tumors per experimental group from 2 independent experiments. D, dual immunofluorescent staining for CD34 and GFP+ pericytes, illustrating that injected pericytes do not incorporate into host blood vessels. (P = pericytes; BV = blood vessels). Immunostaining is representative of multiple sections per mouse. Scale bar = 25 μm.

Pericytes promote aggressive invasion in OVCAR-5 cells in vitro and in vivo

At harvest pericyte co-injected xenografts appeared macroscopically different with indistinct tumor margins indicative of outgrowths. Histological analysis confirmed the presence of invasive nodules of cells at the tumor edges as early as day 6 in the OVCAR-5+P group compared with controls (Fig. 4A).
GFP-immunostaining showed clear encapsulation with GFP+/stromal cells in control tumors, while invasive nodules of GFP+/OVCAR-5 cells were present at the tumor margins of OVCAR-5+/P tumors (Fig. 4B).

Moreover, in vitro Boyden chamber migration assays confirmed that co-culture of OVCAR-5 cells with pericytes increased both migration (2–3-fold; P < 0.05; data not shown) and invasion through Matrigel and an 8-μm filter membrane (Fig. 4C; P < 0.05), while fibroblasts had no significant effect (Fig. 4C).

Pericytes promote aggressive ovarian cancer metastases to distant organs in OVCAR-5 and OVCAR-8 cells

These data led us to examine whether pericytes facilitated metastases in xenografts—BLI analysis of GFP-luciferase+/OVCAR-5 tumors in vivo tracked at regular intervals revealed metastatic spread of ovarian cancer cells to the peritoneal cavity as early as day 21 in OVCAR-5+/P mice (Fig. 4D). By day 28, metastases associated with the intestine, liver, and lung were detected in these mice (Fig. 4E), whereas control mice (OVCAR-5 and OVCAR-5+P injected) were completely free of metastases (Fig. 4E and F). Moreover, a dose-dependent effect on metastatic burden was demonstrable at day 28—increasing the OVCAR-5 cell:pericyte ratio from 10:1 to 1:1 resulted in increased metastases to distant organs (Fig. 4G, P < 0.05), achieving strong statistical significance over OVCAR-5 controls (Fig. 4G; P < 0.001).

By day 42, extensive local metastases were evident throughout the peritoneal cavity associated with the upper and lower gastrointestinal tracts in both control and OVCAR-5+P groups macroscopically (Supplementary Fig. S3A–S3D), and on GFP+ immunostaining (Supplementary Fig. S3E–S3I; day 35), in the pericyte-co-injected group only.

We next tested whether pericytes could affect the less aggressive OVCAR-8 cell line derived from an early-stage cisplatin-treated patient reported to form noninvasive tumors with long periods of...
Co-injection of OVCAR-8 cells with pericytes (10:1 ratio) into nude mice resulted in a 15-day decrease in latency of tumor formation, accelerated tumor growth (Supplementary Fig. S4A), and larger tumor volumes (Supplementary Fig. S4B: \( P < 0.0001 \)). Notably, while GFP-luciferase \(^+\) OVCAR-8 cells did not yield metastases by themselves, pericyte co-injection led to OVCAR-8 metastasis to distal organs, i.e., liver, lung, bladder, kidney, in addition to the GI tract, peritoneum and omentum (Supplementary Fig. S4C–S4I: GFP\(^+\) immunostaining). These data clearly demonstrate the potent ability of pericytes to confer malignancy on nonmetastatic ovarian cancer cells.

Interestingly, bioinformatic analyses of gene expression enrichment in the AOCSc high-grade serous ovarian cancer patients revealed that early-relapse patients identified by a high pericyte score displayed upregulation of molecular pathways, involving matrix degradation, ECM remodeling, negative regulation of cell adhesion, invasion, and migration, compared with those patients with late relapse, using two independent methods (i.e., enrichment analysis of GO terms or KEGG pathways among overexpressed genes or using Gene Set Enrichment Analysis; Supplementary Table S2), providing a clinical correlate for our experimental findings.
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Figure 5.
Pericytes increase recruitment of αSMA⁺ cells to ovarian tumors in mice; increase in αSMA⁺ cells correlates with early relapse in patients with ovarian cancer. A, αSMA staining in OVCAR-5 and OVCAR-5+P tumors at day 11 and day 35. Quantification of total αSMA⁺ cells at day 11 (B) and day 35 (C). Quantification of vessel-associated (D) or stroma-associated αSMA⁺ cells (E) in OVCAR-5 and OVCAR-5+P tumors. Mean ± SEM from 3 independent experiments. F, illustration of vessel-associated (arrows) and stroma-associated (arrowheads) αSMA⁺ cells by co-staining for CD34. Scale bar = 100 μm. G, immunostaining of representative AOCs patient TMA s for αSMA⁺ cells. Scale bar = 200 μm. H, quantification of stroma-associated αSMA⁺ cells in early-, late-, and no-relapse AOCs patient TMA s (n = 7 patients/relapse group; 3 fields/patient). I and J, Kaplan–Meier curves correlating αSMA protein expression and progression-free (I) or overall (J) survival in AOCs serous ovarian cancer patients. K, scatter plot of correlation between expression levels of αSMA and pericyte score from 105 AOCs serous ovarian cancer patients.
Percytes increase recruitment of host αSMA⁺ cells to the TME at sites unrelated to angiogenesis in experimental tumors—also a feature of early-relapse patients with ovarian cancer

An increase in the proportion of the "stromal" compartment of tumors—cells and acellular matrix—is prognostic for poor survival in patients with advanced ovarian cancer (41). We, therefore, immunostained OVCAR-5 xenografts for the stromal cell marker αSMA (Fig. 5A), revealing higher numbers of αSMA⁺ cells in the OVCAR-5-P group at day 11 (P < 0.001) and day 35 (P < 0.05) compared with controls (Fig. 5B and C). Although no quantitative difference in the percentage of vessel-associated αSMA⁺ cells was observed (P = 0.5762; Fig. 5D), a significant increase in stroma-associated αSMA⁺ cells in OVCAR-5-P tumors (P < 0.0001; Fig. 5E) was evident, evaluated by co-staining for αSMA⁺ cells and CD34⁺ blood vessels (Fig. 5F) via immunofluorescence. Notably, the absence of detectable αSMA⁺/Ki67⁺ cells in xenografts even at day 11 (Supplementary Fig. S3A) suggests that this was probably the result of increased recruitment of host αSMA⁺ cells to xenografts, not proliferation.

The accumulation of αSMA⁺ stromal cells was then analyzed in AOCS-patient TMA samples and correlated with clinical outcome; early-relapse ovarian cancer patients (mean PFS time = 8.98 months) showed a significant increase in αSMA⁺ staining compared with late-relapse patients (mean PFS time = 28.45 months; Fig. 5G).

Closely inspection of the αSMA⁺ sections for blood vessels in 7 patient TMA samples per early-, late-, and no-relapse group revealed that this was attributed to increased numbers of tumor stroma-associated αSMA⁺ cells in early-relapse patients (Fig. 5H; P < 0.001), as observed for the OVCAR-5-P experimental tumors, with no significant differences in vessel-associated αSMA⁺ cells (P = 0.4902; data not shown). Thus, our experimental ovarian cancer model strongly mimics the clinical situation with common biological features.

Given that BM-MSCs can be recruited to the TME, we co-stained for αSMA and the murine BM-MSC markers CD73 or Sca-1 in all xenografts. Interestingly, only the pericyte co-injected OVCAR-5 tumors contained CD73⁺ and Sca-1⁺ populations with only a small proportion of αSMA⁺ cells co-expressing these markers (Supplementary Fig. 5B and 5C). Since CAF-derived CXCL12 has been strongly implicated in recruiting BM-MSCs to tumors and driving metastatic spread (42), we immunostained for this chemokine (Supplementary Fig. 5D), not detecting it in the stroma of any experimental tumors, despite abundant CXCL12 expression in OVCAR-5 cells, as reported previously for other ovarian cancer cell lines (43) in all xenografts not correlated with metastasis, suggesting a role for alternate signaling pathways in inducing metastasis, while not excluding a role for CXCL12 in promoting ovarian cancer tumor growth by increasing angiogenesis, as reported previously (43).

Greater αSMA levels predict earlier relapse in serous ovarian cancer patients

We next sought to determine if a single pericyte marker at the protein level could be prognostic at diagnosis. TMA from AOCS serous ovarian cancer patients were immunostained for αSMA and PDGFRβ (and CD34 control), and their expression levels quantitated for individual patients morphometrically and correlated with time to relapse. The levels of CD34 or PDGFRβ expression were not predictive for early-relapse (P = 0.1342, n = 112 patients; and P = 0.1861, n = 102 patients, respectively; Supplementary Fig. S6A and S6B); however, higher levels of αSMA correlated significantly with early relapse for both PFS (P = 0.03691, n = 105 patients; Fig. 5I) and overall survival (P = 0.006652; Fig. 5J). Consistent with this, a significant correlation was observed between αSMA expression levels and pericyte score (Fig. 5K; P = 0.0078), but not CD34 (P = 0.8412) or PDGFRβ (P = 0.3761; Supplementary Fig. S6C and S6D).

Discussion

Percytes are widely known to regulate microvascular function, including structural stability, limiting hypoxia, and blood–brain barrier permeability. In the context of cancers, killing pericytes destabilizes tumor vasculature, resulting in tumor regression (27), or causes hypoxia, inducing EMT and increased metastatic dissemination in various cancers (35). Our data demonstrate that placing pericytes in the tumor stroma of OVCAR-5 and -8 ovarian cancer cells while leaving the tumor vasculature intact results in accelerated tumor expansion via increased cell proliferation, shortening the latency of OVCAR-8 tumors by 15 days. Moreover, pericytes induced invasion and metastatic spread in nonmetastatic OVCAR-8 cells—a core clinical feature of aggressive serous ovarian cancer (1, 44, 45), and faster, distal spread of OVCAR-5 cells compared with controls that metastasized only locally within the peritoneal cavity to the gastrointestinal tract. These data demonstrate that normal MSC-like pericytes placed in close proximity to ovarian cancer cells drive malignant conversion, while normal fibroblasts do not affect tumor growth or metastasis, as reported previously. Notably, this was observed despite the use of heterologous, that is, non-ovarian stromal cells (primarily due to the difficulties in obtaining human ovarian tissue in sufficient quantities and at regular frequencies to undertake adequate experimentation), indicating sufficient conservation of function exists in MSC-like pericytes, despite being tissue of origin, consistent with published data (24). Indeed, current transcriptional profiling work in our laboratory comparing adult and neonatal pericytes from male and female donors and from different anatomical sites reveals minimal differences in mRNA expression profiles. We speculate that pericytes are a more potent stromal stem–cell–like population than fibroblasts, whose involvement is a harbinger for poor clinical outcome in patients.

Consistent with this notion, we demonstrated that the pericyte signature had strong clinical relevance for high-grade serous ovarian cancer patients—outperforming the stromal signature derived from ovarian cancer patient stroma (5) in predicting significantly earlier patient relapse, despite similar treatment in both the AOCS (n = 215) and the NCI TCGA patient datasets (n = 408). The early-relapse patient group expressed gene sets enriched for biological processes clearly increased experimentally by pericytes such as invasion and migration that are key features of aggressive metastatic disease, that is, cell motility, negative regulation of cell adhesion and, EMT. In contrast, the inability of normal fibroblasts to promote malignant ovarian cancer progression was correlated well with their signature performing relatively poorly as a predictor of early patient relapse. These data illustrate the need to understand the nature of stromal heterogeneity in both normal and cancerous tissues. The ability of tumor cells to attract specific subtypes of stromal cells may facilitate tumor progression to a malignant state. Presumably, the process of pericyte association and dissociation from blood vessels during tissue remodeling in wound healing and cancer requires tight molecular regulation. The contribution of pericytes to malignant...
progression has remained unappreciated, masked by the fact that the markers used to detect "CAFs" or BM-MSCs are also co-expressed by pericytes, e.g., αSMA, MCAM/CD146, and CD73. A further potentially confounding factor is the low incidence at which these cells may exist in the TME—like most stem cell populations, a large number is not required to effect significant change.

Apart from their ability to affect tumor cell proliferation and invasive capacity directly in co-culture Transwell assays in vitro, also mirrored in xenografts in vivo, the most striking feature of the pericyte co-injected tumors was the recruitment of host αSMA+ yet Ki67- cells that formed a nonvascular network between the OVCAR-5 tumor cells in early day 11 xenografts. The correlation with increased αSMA+ cell numbers in TMA's from early-relapse patients suggested that this was a critical functional component of the TME, indicative of tumor progression, leading to the finding that αSMA protein levels yield prognostic significance in a large sample of patient TMA's. However, the combined pericyte signature at the mRNA level was much more effective at predicting relapse \((P = 0.00067)\) than the level of αSMA staining \((P = 0.03691)\). High αSMA protein has also been reported to be of prognostic value in colorectal cancer \((7)\) and at the mRNA level in pancreatic adenocarcinoma \((8, 46)\). In contrast, recent studies in murine models of pancreatic adenocarcinoma provide evidence in favor of αSMA+ cells having a role in limiting tumor growth and metastasis by either suppressing immune surveillance \((47)\) or perhaps decreasing tumor angiogenesis \((48)\). Notably, in pancreatic adenocarcinoma patients low αSMA levels were associated with poorer survival \((47)\). These studies further substantiate the need to examine the intratumoral heterogeneity of αSMA+ stromal subsets and examine their role in epithelial cancers of different tissue origins (e.g., ovarian vs. pancreas).

Interestingly, fibroblast co-injected OVCAR-5 tumors did not show a sustained increase in the number of αSMA+ cells \(\text{(data not shown)}\), correlating with unchanged tumor growth, absence of invasive cells at tumor margins, and absence of metastasis. Whereas the influx of higher αSMA+ cell numbers in both experimental tumors and early-relapse patient TMA's may be an attempt by the host to limit tumor growth and therefore a red herring, it remains possible that their recruitment or perhaps a subtype therein is required for metastatic spread. Another major difference in pericyte co-injected OVCAR-5 tumors was the recruitment of host Sca-1+/CD73+ BM-MSCs not observed in OVCAR-5 controls or fibroblast co-injected tumors—given their widely reported role in cancer cell dissemination, their recruitment by pericytes may well contribute to metastasis.

The inability of the normal pericyte marker PDGFRβ to subset serous ovarian cancer patients for the probability of relapse suggests that αSMA and PDGFRβ do not identify pericytes exclusively in cancer and are expressed by other stromal cells in the TME. Consistent with this, PDGFRβ expression was observed in the tumor stroma in addition to its classic perivascular localization in patient TMA's. Attempts to define a single pericyte marker to predict poor prognosis in patients with ovarian cancer at diagnosis were only partially successful. Poor correlation between high pericyte score and PDGFRβ expression levels in TMA's belied its inability to predict relapse with a high degree of certainty.

CD34 served as a negative control, given poor correlation between MVD and early versus late relapse. Thus, although angiogenesis is obviously critical for tumor development, it is not relevant to malignant progression at advanced stages of malignancy predomnantly in the patients analyzed here. Although αSMA protein levels achieved reasonable significance levels for predicting relapse \((P = 0.03691)\), it is likely that a number of pericyte markers might be required to identify patients at greater risk of relapse.

An obvious target of further work is to understand the process by which pericytes become dissociated from blood vessels during physiological tissue remodeling. We speculate that cyto-kines used by endothelial cells to attract pericytes to newly forming blood vessels such as PDGF-B may also be synthesized by tumor cells—including, overexpression of PDGF-B in squamous carcinoma models promotes tumor cell proliferation and acts as a chemoattrant and activator for mesenchymal cells \((49)\).

However, metastases were not observed in this model, suggesting that this single factor is unlikely to cause malignant progression. Certainly the mRNAs co-expressed by pericytes and early-relapse ovarian cancer patients point to a coordinate regulation of genes enriched in processes essential for tissue remodeling.

These data represent a paradigm shift in the current thinking about the contribution of pericytes to the TME while providing an effective means of identifying those patients that are at significantly greater risk of earlier relapse and mortality. Undoubtedly, this brings a further level of complexity to therapeutic approaches aimed at inhibiting angiogenesis, but provides new opportunities to develop effective strategies against stem-cell–like pericytes in the TME, given that anti-pericyte reagents not only exist, but are in clinical use in the guise of anti-angiogenic therapies. The in vitro invasion data suggest that pericytes secrete soluble factors that induce tumor cell dissemination forming the basis for identifying specific proteins that promote malignant progression that could also serve as biomarkers for ovarian cancer, particularly early-stage disease, given that experimentally, pericyte involvement in the TME results in the induction of metastases in the poorly tumorigenic and nonmetastatic OVCAR-8 cells. Perhaps the greatest barrier to translating the significance of our findings to early diagnosis and thereby increasing the chances of overall patient survival is the lack of early-stage ovarian cancer patient databases combining transcriptional and proteomic profiling with clinical outcome following diagnosis. The collation of patient cancer proteomic analysis being undertaken by the NCI CTIAC initiative is eagerly anticipated, given the corroboration of our findings between the AOCS and TCGA patient datasets.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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## Pericytes Promote Malignant Ovarian Cancer Progression in Mice and Predict Poor Prognosis in Serous Ovarian Cancer Patients

Devbarna Sinha, Lynn Chong, Joshy George, et al.


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