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Stem-Like Ovarian Cancer Cells Can Serve as Tumor Vascular Progenitors

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ABSTRACT

Neovascularization is required for solid tumor maintenance, progression, and metastasis. The most described contribution of cancer cells in tumor neovascularization is the secretion of factors, which attract various cell types to establish a microenvironment that promotes blood vessel formation. The cancer stem cell hypothesis suggests that tumors are composed of cells that may share the differentiation capacity of normal stem cells. Similar to normal stem cells, cancer stem cells (CSCs) have the capacity to acquire different phenotypes. Thus, it is possible that CSCs have a bigger role in the process of tumor neovascularization. In this study, we show the capacity of a specific population of ovarian cancer cells with stem-like properties to give rise to xenograft tumors containing blood vessels, which are lined by human CD34+ cells. In addition, when cultured in high-density Matrigel, these cells mimic the behavior of normal endothelial cells and can form vessel-like structures in 24 hours. Microscopic analysis showed extensive branching and maturation of vessel-like structures in 7 days. Western blot and flow cytometry analysis showed that this process is accompanied by the acquisition of classic endothelial markers, CD34 and VE-cadherin. More importantly, we show that this process is vascular endothelial growth factor–independent, but IKKβ-dependent. Our findings suggest that anti-angiogenic therapies should take into consideration the inherent capacity of these cells to serve as vascular progenitors. STEM CELLS *2009;27:2405–2413*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neovascularization is necessary for tumor maintenance and progression. During embryogenesis and development, the formation of vascular networks occurs via two non-mutually exclusive events: angiogenesis, the formation of new vascular networks from pre-existing blood vessels; and vasculogenesis, the de novo production of endothelial cells from precursor cells. Angiogenesis has been the prevailing concept used to describe the process of neovascularization within the tumor microenvironment [1, 2]. However, the demonstration that circulating bone marrow-derived endothelial progenitor cells can be recruited to the tumor site and can differentiate into endothelial cells suggested that vasculogenesis may also contribute to tumor vascularization [3]. The most described contribution of the cancer cells in tumor neovascularization is the secretion of pro-angiogenic factors that attract various cell types into the tumor bed and actively establish a microenvironment that promotes blood vessel formation [4, 5]. As such, the up regulation of vascular endothelial growth factor (VEGF) secretion by cancer cells is the main target of most anti-angiogenic therapies [1].

The cancer stem cell hypothesis suggests that tumors are maintained by a small population of cells that are chemo-resistant and can therefore persist during treatment, eventually rebuild the tumor, and lead to recurrence [6, 7]. Cancer stem cells (CSCs) share numerous properties with normal stem cells in that they are able to self-renew and differentiate into other phenotypes. We hypothesize that this property of CSCs may confer the ability, under specific conditions (i.e., hypoxia), to differentiate into an endothelial cell phenotype and hence allow them to actively contribute to the neovascularization process by serving as vascular progenitors [8].

Our group recently reported that the cell surface marker CD44 can enrich for a sub-population of ovarian cancer cells with stem-like properties [9]. We demonstrated that these cells, referred to from here on as Type I epithelial ovarian cancer (EOC) cells: (1) are tumorigenic and can recapitulate the heterogeneity of the original tumor; (2) can form self-renewing spheroids; (3) have high levels of stem cell markers

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β-catenin, Oct-4, and SSEA-4; (4) have constitutively active IKKβ/NF-κB; (5) constitutively secrete IL-6, IL-8, MCP-1, and GRO-α; and (6) are chemo-resistant [9].

Subcutaneous injection of this cell population in Athymic Nude Mice (NcR) nude mice resulted in highly vascular tumors. Using this animal model and an in vitro 3D model, we sought to demonstrate that Type I EOC cells can serve as vascular progenitors. In this study, we report that xenografts derived from primary cultures of Type I EOC cells contained functional blood vessels lined by CD34-positive ($CD34^+$) cells of human origin. Moreover, we show that Type I EOC cells, but not mature ovarian cancer cells (Type II EOC cells), are able to form vessel-like structures in vitro, reminiscent of normal endothelial cells. More importantly, we show that Type I EOC cells are able to acquire endothelial-specific markers during the process of vessel formation. Finally, we provide evidence that this process is independent of VEGF but dependent on IKKβ.

Materials and Methods

Cell Lines and Culture Conditions

Primary cultures of ovarian cancer cells were isolated from patient ascites or tumor tissue and cultured as previously described [9–11]. $CD44^+$ cells were isolated using a two-step process: staining with anti-CD44-FITC (eBioscience, San Diego, CA, USA, http://www.ebioscience.com) and isolation using anti-FITC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), both according to manufacturers' instructions. For the studies described below, we used a panel of three $CD44^+$ and three $CD44^-$ established cell lines and three $CD44^+$ and three $CD44^-$ freshly isolated cells that were used immediately after isolation. HEECs were cultured as previously described [12]. The use of patients' samples was approved by the Yale University's Human Investigations Committee.

Establishment of Tumors In Vivo

The Yale University Institutional Animal Care and Use Committee approved all in vivo studies described. Subcutaneous tumors were established in NCR nude mice as previously described [9].

Immunohistochemistry

Sections (5 μm) were deparaffinized in Histosolve and rehydrated. Antigen retrieval was performed using pre-warmed Target Retrieval Solution (Dako USA, Carpinteria, CA, USA, http://www.dakousa.com) in a steamer for 30 minutes. Primary antibodies were applied to slides for 20 minutes at room temperature. Primary antibodies used were human-specific CD34 (1:50) and mouse-specific CD31 (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, http://www.scbt.com), according to manufacturer's instruction. These antibody concentrations yield strong staining when used in positive controls and low background when used in negative control samples. Positive and negative controls are described in the figure legends. CD31 and CD34 sections were developed using DAB and Fast Red, respectively (Envision Double Stain System; Dako USA). Slides were counterstained with Hematoxylin and mounted with Aqueous Mounting Medium (Dako USA).

Matrigel Differentiation

Cells ($5\text{-}10 \times 10^4$) were plated in BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Diego, CA, USA, http://www.bdbiosciences.com) in the presence or absence of sFlt-1 (R&D Systems Inc., Minneapolis, MN, USA, http://www.rndsystems.com) or BAY 11-7082 (Sigma-Aldrich, St. Louis, MO, USA, http://www.sigma-aldrich.com). Vessel formation was monitored using the Incucyte real-time video imaging system (Essen Instruments, Ann Arbor, MI, USA, http://www.essen-instruments.com).

Flow Cytometry

Flow cytometry analysis was performed as previously described [9]. Briefly, cells were either trypsinized or recovered from Matrigel using BD Cell Recovery Solution (BD Bioscience) according to manufacturer's instructions. Pelleted cells were incubated with either PE-anti-CD34 or APC-anti-CD133 (eBioscience). Data was acquired using BD FACSCalibur and analyzed using Cell Quest Pro (BD Bioscience).

Western Blot Analysis

SDS-PAGE and western blots were performed as previously described [13, 14]. The following antibodies were used: anti-VE-cadherin (1:1,000; Cell Signaling Technology, Beverly, MA, USA, http://www.cellsignal.com), anti-CD44 (1:5,000; Abcam, Cambridge, U.K., http://www.abcam.com), anti-vascular endothelial growth factor receptor (VEGFR)-2 (1:500; Cell Signaling Technology), and anti-Actin (1:10,000; Sigma-Aldrich).

Measurement of Cytokines/Chemokines

Levels of IL-6, IL-8, MCP-1, and VEGF were measured in cell-free supernatants, from either monolayer or Matrigel, after 72h of culture using the Bioplex Pro Cytokine Assay (Bio-Rad, Hercules, CA, USA, http://www.bio-rad.com). Data were acquired using the Bioplex system (Biorad) and analysis was performed using the Bioplex software as previously described [9, 15]. Protein standards provided in the kit served as positive control and culture media (without cells) were used as negative control. Data shown are the mean of three independent experiments.

Results

Mouse Xenografts from Pure Type I EOC Cells Express $CD34^+$ Cells of Human Origin

Type I EOC cells are characterized by the constitutive secretion of high levels of pro-angiogenic cytokines and chemokines such as IL-8, MCP-1, and GROα [14, 15]. This suggests that this cell population might promote or enhance the process of tumor neovascularization. To evaluate this, we established a subcutaneous xenograft of pure human Type I EOC cells in NCR nude mice as previously described [9]. Once the tumors were ~8-10 mm in diameter, mice were sacrificed and tumors were excised. Morphological assessment of the xenografts showed significant vascularization surrounding the tumor mass (Fig. 1A) suggesting the recruitment of host blood vessels and promotion of angiogenesis. Thus, to determine the presence of mouse-derived endothelial cells in the xenograft, we immunostained paraffin sections of the tumors with a specific anti-mouse CD31 antibody. Our results showed that only a few blood vessels in the xenograft stained positively for CD31 and that most of these vessels were located in the periphery of the tumor (Fig. 1B). The majority of the tumor blood vessels, especially those in the center of the tumor, were CD31-negative and were, therefore, not of mouse origin (Fig. 1C).

To determine the origin of the CD31-negative blood vessels we immunostained the xenograft sections with a specific anti-human CD34 antibody. Interestingly, numerous $CD34^+$ endothelial cells were observed lining the blood vessels of these tumors (Fig. 1D–1F). This suggests that most of the CD31-negative blood vessels contain endothelial cells originating from the human Type I EOC cells used to establish the subcutaneous tumor. Similar to the observation that not all blood vessel walls were stained with the CD31 antibody, some blood vessels were also negative for CD34 (Fig. 1E). We did not observe any mosaic vessels; this suggests that in

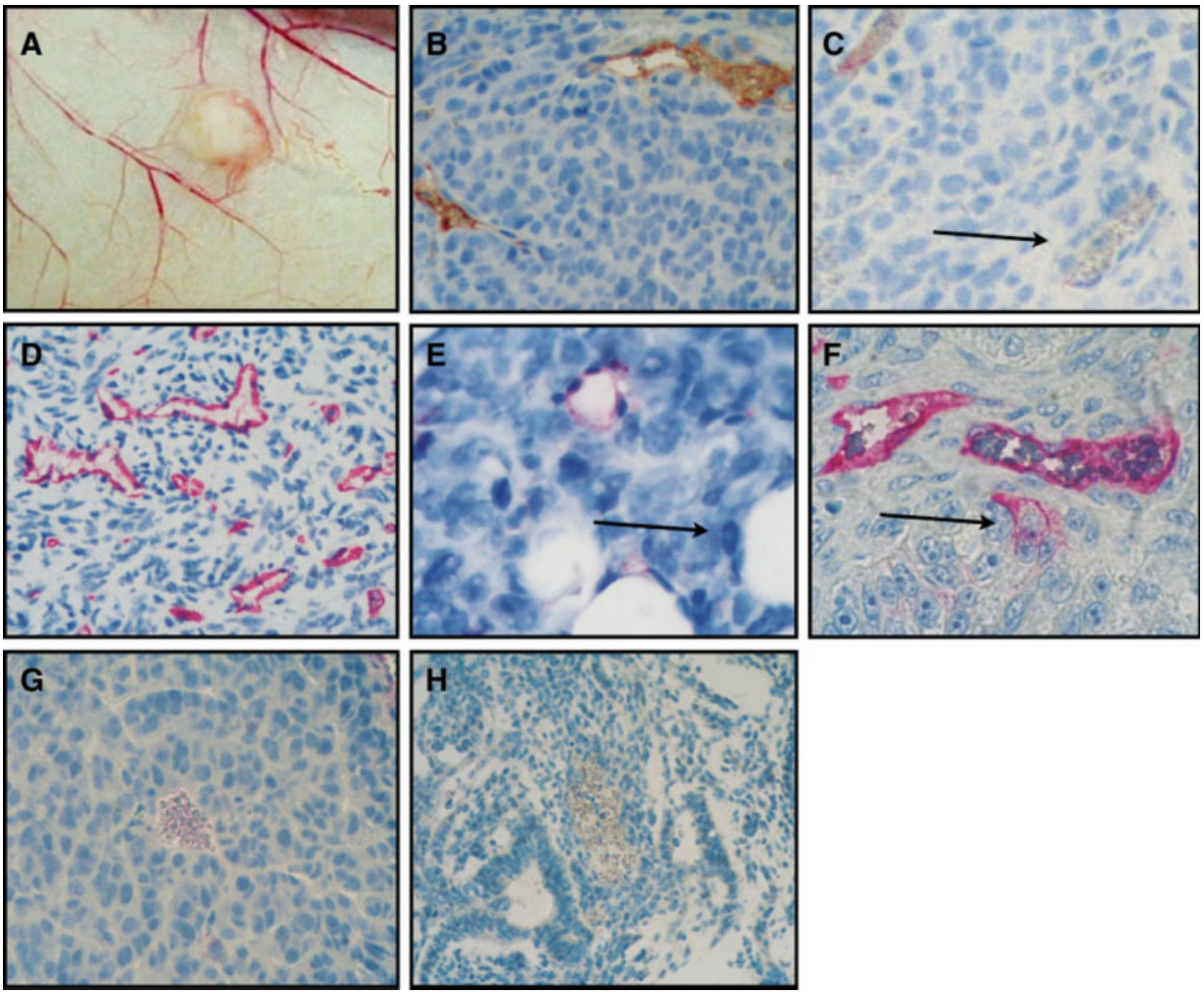

Figure 1. Xenograft tumors obtained from Type I EOC cells contain both $CD34^+$ (human derived) and $CD31^+$ (mouse derived) blood vessels. **(A)** Subcutaneous tumors obtained from human ovarian cancer cells with stem-like properties show recruitment of mouse blood vessels. Pure population of Type I EOC cells was injected subcutaneously in NCR mice. Mice were sacrificed when tumors reached ~8–10 mm. Note the projection of host blood vessels towards the human-derived tumor. **(B–C)** Immunohistochemistry analysis of paraffin sections from xenografts using anti-mouse CD31 antibody. Arrow in **C** shows CD31-negative blood vessel. **(D–F)** Immunohistochemistry analysis of paraffin sections from xenografts using anti-human CD34. Arrow in **E** shows CD34-negative blood vessel; arrow in **F** shows $CD34^+$ cancer cell. **(G)** Human ovarian cancer tumor stained with mouse anti-CD31. **(H)** Mouse endometrium stained with human anti-CD34. Representative figures of at least six independent experiments, $n = 6$ per experiment.

the xenografts, blood vessels were either entirely of mouse or of human origin.

Interestingly, we observed the presence of $CD34^+$ cancer cells near some blood vessels (Fig. 1F). Although they clearly stain for CD34, this staining was less intense than those found in cells that line the blood vessel walls. More importantly, however, these cells have a different morphology than the $CD34^+$ cells that line the blood vessels. Whereas the $CD34^+$ cells lining the blood vessels are thinned-out, characteristic of endothelial cells, the $CD34^+$ cells located in the periphery of the blood vessels are rounded and morphologically similar to Type I EOC cells [16]. Taken together, these results suggest that many of the endothelial cells observed in the blood vessels of the xenograft tumor originated from the human Type I EOC cells.

Type I, but Not Type II, EOC Cells Can Form Vessel-Like Structures In Vitro

The presence of $CD34^+$ cells in the blood vessels of xenograft tumors suggests that the endothelial cells originated from the human Type I EOC cells. This also suggests that Type I EOC cells may have the capacity to differentiate into endothelial cells. To further evaluate this hypothesis, we used a 3D in vitro system using high density Matrigel, which is widely used to study endothelial cell differentiation and function [17]. As positive control for this system, we used normal endothelial cells isolated from human endometrium (HEECs) [18]. As shown in Figure 2A, HEECS grown in Matrigel are able to form vessel-like structures as previously described [12]. However, when Type I or Type II EOC cells were grown in Matrigel, only the Type I EOC cells were able to form vessel-like structures similar to those observed with HEECS (Figs. 2B–2E). In contrast, Type II EOC cells, obtained from the same patient, formed clusters in the Matrigel (Fig. 2F). Type I EOC cells begin to form these structures 2 hours after plating and completed tubes were observed around 24 hours (supporting information Fig. 1). Further maturation and branching of the vessel-like structures can be observed 7 days after plating in Matrigel (Fig. 2D–2E). The sequence of events leading to the tube formation was identical between normal endothelial cells and

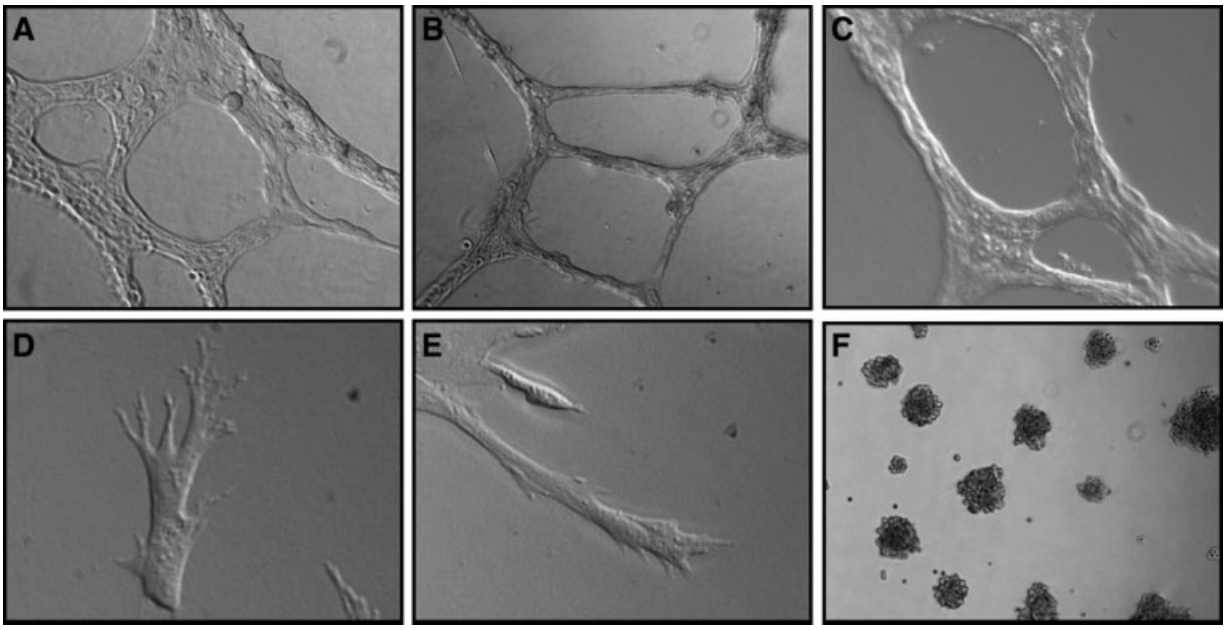

Figure 2. Differentiation of Type I EOC cells into vessel-like structures in vitro. **(A)** Human endometrial endothelial cells, **(B–E)** Type I EOC cells, and **(F)** Type II EOC cells were plated in high-density Matrigel and vessel formation was monitored for 72 hours. Note the maturation of branches in **D** and **E**. Representative figures of three cell types in each group. Each experiment was repeated at least three times.

Type I EOC cells as demonstrated by live video imaging (supporting information Video 1 and Video 2).

These data demonstrate that when grown in Matrigel, only Type I EOC cells can differentiate into vessel-forming cells, which mimic the same structures formed by normal HEECs. Type II cells cannot.

Type I EOC Cells Do Not Express the Classical Markers of Endothelial Progenitor Cells

The capacity of Type I EOC cells to form vessel-like structures in vitro and the presence of $CD34^+$ cells in the xenograft tumors suggests that they may serve as endothelial progenitor cells (EPCs). Classical EPCs are bone-marrow derived and have been characterized as being $CD34^+/CD133^+$ [19]. To determine if the primary cultures of Type I EOC cells used to form the xenografts have any contaminating bone-marrow derived EPCs, we analyzed the expression of these markers by flow cytometry. Figure 3 shows that the Type I cultures are negative for both CD34 and CD133. This provides more evidence that the $CD34^+$ cells lining the blood vessels in the xenograft were derived from the Type I EOC cells and not from any contaminating bone-marrow derived EPCs.

Type I EOC Cells Acquire Endothelial Cell Markers After Differentiation

The demonstration that Type I EOC cells could form vessel-like structures in Matrigel, very similar to those formed by HEECs, suggested that they may have the capacity to undergo differentiation into endothelial cells. To conclusively show that Type I EOC cells are able to acquire an endothelial cell phenotype upon differentiation, Type I or II EOC cells were grown in monolayer or in Matrigel for 72 hours and analyzed for the expression of endothelial-specific markers by Western blot and flow cytometry. Figure 4 shows that both Type I and II EOC cells grown in monolayer do not express the endothelial marker VE-cadherin. Following differentiation in Matrigel, however, only Type I EOC cells acquired VE-cadherin. Flow cytometry analysis showed that in addition to gaining VE-cadherin expression, Type I EOC cells also gained CD34 expression after Matrigel differentiation (Fig. 3B). Interestingly, Type I EOC cells did not lose the stem cell marker CD44 upon differentiation (Fig. 4). These results further confirm that Type I EOC cells have the capacity to differentiate into endothelial cells and acquire endothelial cell phenotype.

Matrigel-Differentiated Type I EOC Cells Secrete Lower Levels of Cytokines

We previously reported that Type I EOC cells constitutively secrete high levels of IL-6, IL-8, and MCP-1 [14, 15]. Our next objective was to determine the effect of differentiation on cytokine secretion. Thus, we collected the supernatants from Type I or II EOC cells grown in monolayer or Matrigel for 72 hours and measured the levels of cytokines and chemokines. For Type I EOC cells, our results show that upon Matrigel differentiation, MCP-1 secretion was not significantly decreased ($p = .07$) but the secretion of IL-6 and IL-8 was inhibited significantly ($p = .03$ and .01, respectively) (Fig. 5). More importantly, analysis of VEGF levels showed that this cytokine was also significantly down-regulated upon Type I EOC cell differentiation ($p = .01$). For the Type II EOC cells, Figure 5 shows that when grown in monolayer, these cells secrete undetectable amounts of IL-6, IL-8, and MCP-1 as previously reported [14, 15] and that the levels of these cytokines/chemokines were unaffected upon culturing in Matrigel. Interestingly, Type II EOC cells secrete higher levels of VEGF than Type I EOC cells ($p = .005$). VEGF levels were, however, unchanged when Type II EOC cells were grown in Matrigel ($p = .1$). Taken together, these results suggest that on the acquisition of endothelial cell markers, Type I EOC cells lose the capacity to constitutively secrete high levels of IL-6, IL-8, and VEGF. Moreover, it suggest that in vitro vessel formation may not require VEGF.

Capacity to Form Vessel-Like Structures Is Inherent to Type I EOC Cell Phenotype and Does Not Depend on Factors Secreted by Type I EOC Cells

The high-density Matrigel that we used in the experiments described above contains several growth factors. To determine

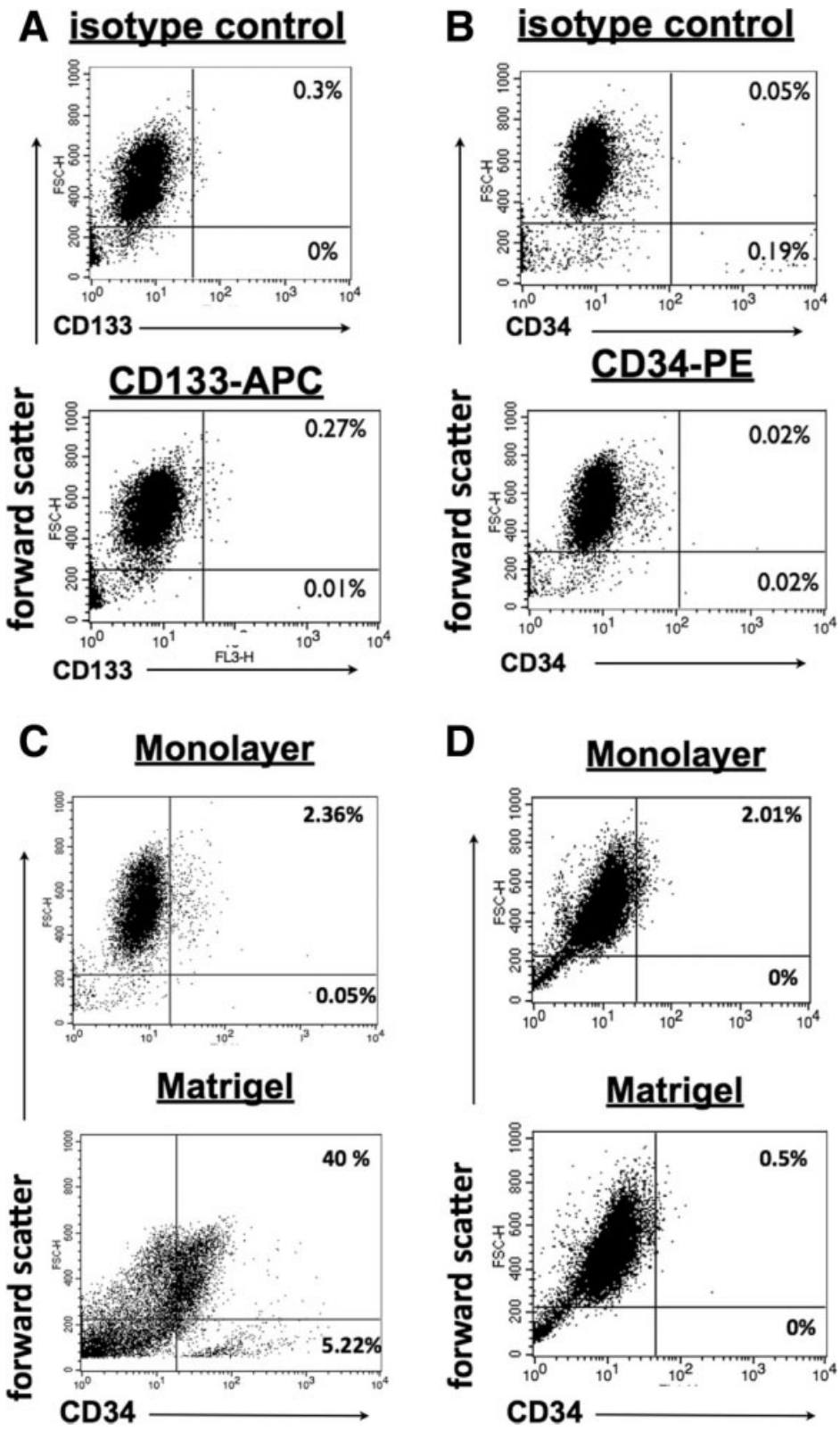

Figure 3. Type I EOC cells do not express the endothelial precursor cell markers CD133 and CD34 but acquired CD34 after differentiation. Cultures of Type I EOC cells grown in monolayer were analyzed for the expression of **(A)** CD133 and **(B)** CD34 by flow cytometry. Note that the cells are negative for the two markers. **(C)** CD34 levels were compared in Type I EOC cells grown for 72 hours either in monolayer or Matrigel. A significant increase on $CD34^+$ cells is observed on differentiated Type I EOC cells. **(D)** CD34 levels were compared in Type II EOC cells grown for 72 hours either in monolayer or Matrigel. The CD34 antigenic shift was not observed in Type II EOC cells. Data shown represent results from three independent experiment with at least three different cell cultures.

if these factors are likewise required for Type I EOC cell vessel formation, Type I EOC cells were cultured in growth factor-reduced Matrigel. A comparison of growth factor levels in high-density Matrigel and reduced-growth factor Matrigel is summarized at http://www.bdbiosciences.com/discovery_labware/products/display_product.php?keyID = *230*. As shown in Figure 6I, these growth factors seem to be required for HEEC in vitro vessel formation. In contrast, Type I EOC cells maintained the capacity to form vessel-like structures even when plated in a low growth factor environment (Fig. 6B).

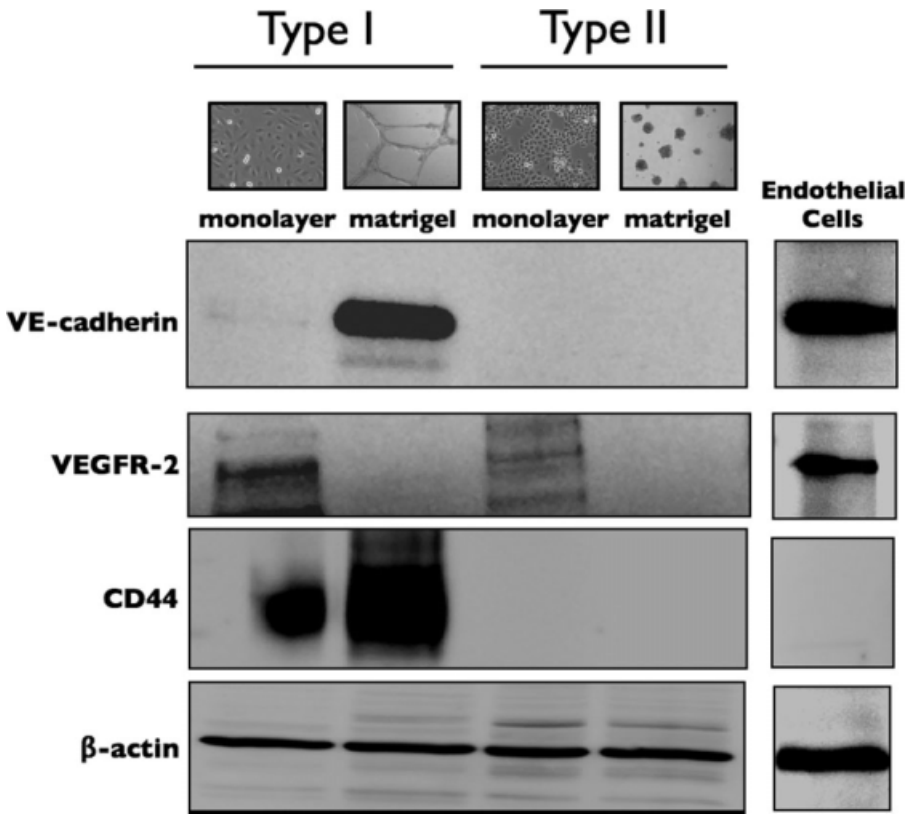

Figure 4. Type I EOC cells acquire endothelial cell markers following differentiation in Matrigel. Cells were grown for 72 hours either as a monolayer or in Matrigel and cell lysates analyzed by western blot for the endothelial-specific marker VE-cadherin, the stem cell marker CD44, and vascular endothelial growth factor receptor-2. Endothelial cells serve as positive control.

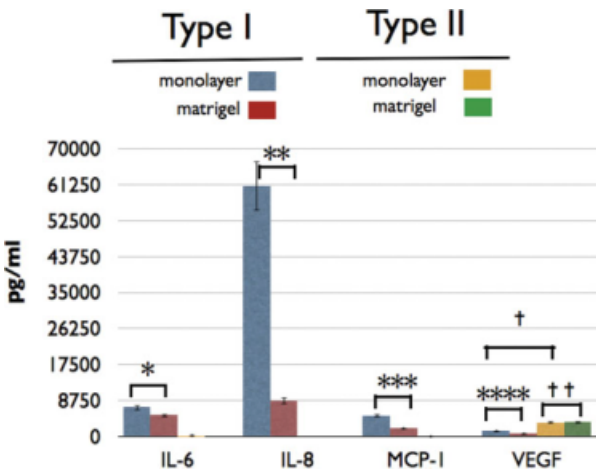

Figure 5. Levels of cytokines secreted by Type I or II EOC cells cultures in monolayer or Matrigel. Cultures were grown for 72 hours either as monolayer or in Matrigel and cell-free supernatants were used to measure the levels of cytokines/chemokines as described in the Materials and Methods. *$p = .03$; **$p = .01$; ***$p = .07$; ****$p = .01$; †$p = .005$; ††$p = .01$.

This suggests that the capacity of Type I EOC cells to form vessel-like structures is independent of these growth factors.

As mentioned above, Type I EOC cells constitutively secrete the pro-angiogenic factors IL-8 and GROα; therefore, our next objective was to determine if vessel formation is a result of the paracrine effects of these factors. Thus, Type II EOC cells were grown in Matrigel with increasing concentrations of conditioned media (CM) obtained from Type I EOC cells. After 72 hours of incubation, the presence of CM from Type I EOC cells did not induce vessel formation in Type II EOC cells (Fig. 6G). This suggests that the ability to form vessel-like structures is inherent to the cellular phenotype of Type I EOC cells and does not depend on the factors it secretes.

We also plated Type I EOC cells in Matrigel with increasing concentrations of CM from Type II EOC cells. Figure 6C shows that Type I EOC cells maintained the capacity to form vessel-like structures even in the presence of CM from Type II EOC cells. This suggests that CM from Type II EOC cells do not contain any factors that can inhibit the formation of the vessel-like structures. Taken together, these results suggest that the capacity to differentiate is inherent to the Type I EOC cell phenotype and independent on external growth factors.

Vessel Formation by Type I EOC Cells Does Not Require VEGF

The VEGF family of proteins is one of the most potent inducers of angiogenesis and vasculogenesis [1, 4] and has been the target for the treatment of numerous type of solid cancers [1, 5]. Our results above showed that VEGF levels decreased upon Type I EOC cell differentiation in Matrigel (Fig. 5). Moreover, western blot analysis for VEGFR-2 showed that Type I EOC cells grown in monolayer express VEGFR-2 but lost its expression following vessel formation (Fig. 4). Interestingly, Type II EOC cell also expressed VEGFR-2, although at lower levels, and also completely lost its expression once cultured in Matrigel.

To determine the role of VEGF on Type I EOC cell vessel formation, cells were cultured in the presence or absence of the soluble VEGFR-1 inhibitor, sFLT-1. Thus, Type I EOC cells and HEECs were grown in Matrigel with or without 1 μg/ml sFLT-1. Results with HEEC showed inhibition of vessel formation (Fig. 6J) similar to those observed with Human Umbilical Vein Endothelial Cells (HUVECs) treated

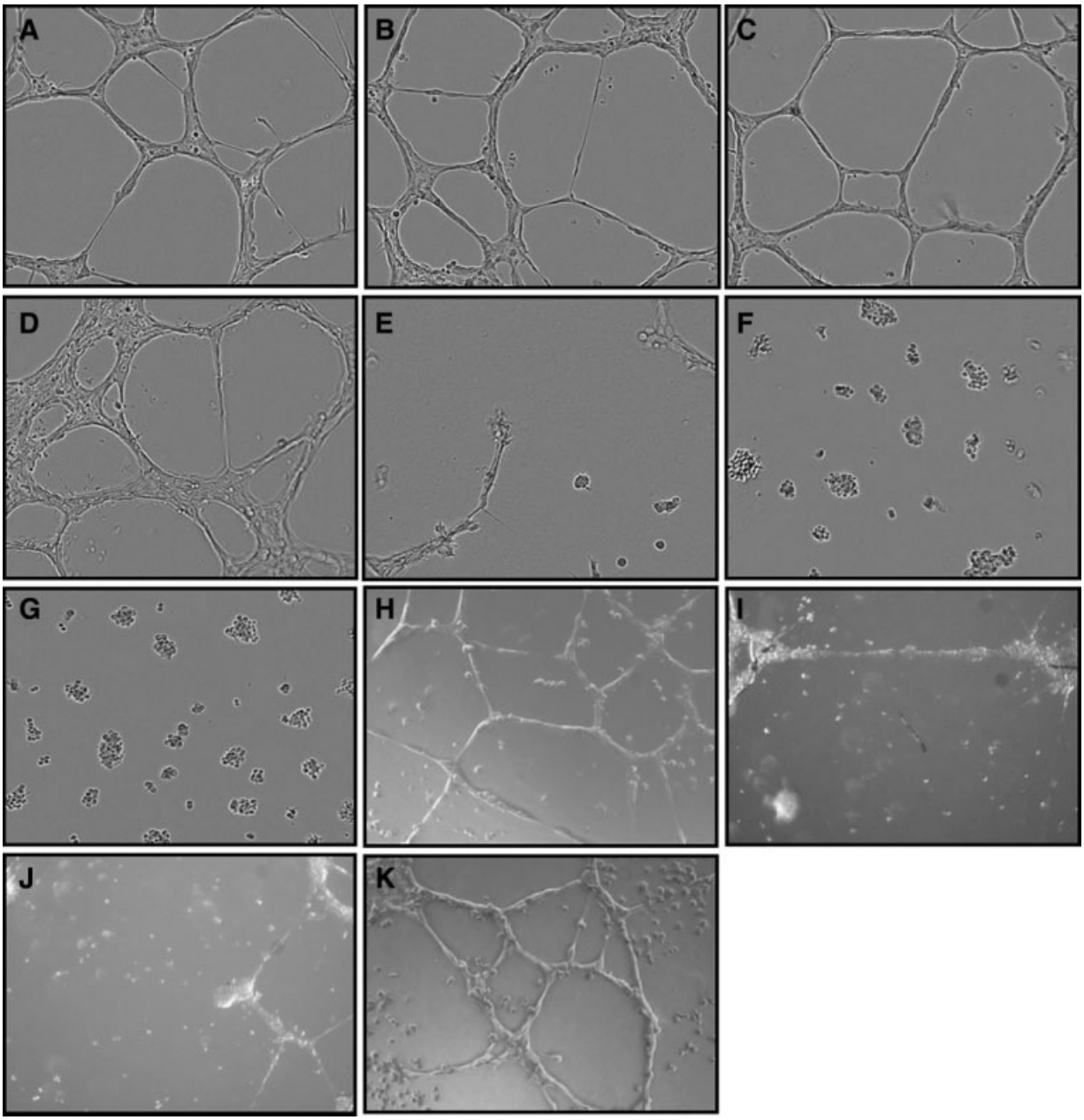

Figure 6. Type I EOC cell vessel formation is independent of external growth factors but dependent on IKKβ. **(A–E)** Type I EOC cells in Matrigel: control, in growth factor-reduced Matrigel, in 50% Type II conditioned media, with 1 μg/ml sFlt-1, and with 0.4 μm BAY 11-7082, respectively; **(F–G)** Type II EOC cells in Matrigel: control and in 50% Type I conditioned media, respectively; **(H–K)** normal human endometrial endothelial cells in Matrigel: control, in growth factor-reduced matrigel, with 1 μg/ml sFlt-1, with 0.4 μm BAY 11-7082, respectively.

with the same dose of sFLT-1 [20]. In contrast, Type I EOC vessel formation was not affected by sFlt-1 at the same concentration (Fig. 6D). This result suggests that the capacity of Type I EOC cells to form vessel-like structures does not require VEGF and that this event has different molecular players than the process of vessel formation by HEECs.

Vessel Formation by Type I EOC Cells Requires IKKβ

We previously showed that Type I EOC cells have a constitutively active IKKβ/NF-κB pathway [15], which is responsible for the constitutive cytokine production [14]. Since Type I EOC cell vessel formation does not require VEGF, our next objective was to determine whether this process is under the control of IKKβ. Thus, Type I EOC cells were plated in Matrigel in the presence or absence of the IKKβ inhibitor, BAY 11-7082 [21]. Figure 6E shows that the inhibition of IKKβ significantly inhibited vessel formation by Type I EOC cells but had no effect on vessel formation by HEECs (Fig. 6K).

Discussion

We demonstrate for the first time that Type I EOC cells have the capacity to acquire an endothelial cell phenotype.

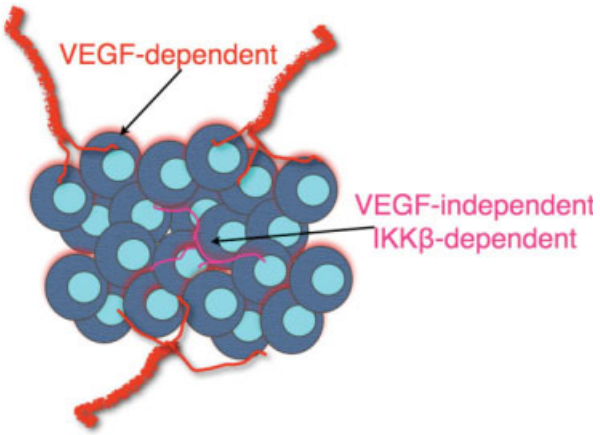

Figure 7. Model showing contribution of Type I EOC cells in tumor neovascularization. We propose that in ovarian cancer, neovascularization can occur through classic vascular endothelial growth factor (VEGF)-dependent angiogenesis and, more importantly, through a VEGF-independent but IKKβ-dependent differentiation of Type I EOC cells.

Specifically, we showed that $CD44^+$/VE-$cadherin^-$/$CD34^-$ Type I EOC cells can differentiate into a $CD44^+$/VE-$cadherin^+$/$CD34^+$ phenotype after vessel formation in Matrigel. In addition, we showed that this differentiation process is inherent to the phenotype of Type I EOC cells, does not require VEGF, but involves IKKβ. More importantly, we demonstrate that xenograft tumors from a pure population of Type I EOC cells contain endothelial cells of human origin. Taken together, these results provide evidence that, in addition to secreting pro-angiogenic factors, Type I EOC cells can directly contribute to tumor neovascularization by acting as building blocks of the vessel wall.

Tumor neovascularization has been extensively demonstrated to result from the process of angiogenesis [1]. This process involves the "sprouting" of new vasculature from mature and existing blood vessels and is controlled by the balance between pro-angiogenic factors and angiogenic inhibitors. During the process of tumor angiogenesis, the role ascribed to the cancer cells has been that of an initiator of "angiogenic switch," a critical step in tumor progression [22, 23]. Numerous studies have shown that cancer cells secrete pro-angiogenic factors such as VEGF, Ang-1, and Ang-2 [24, 25], which promote the formation of new blood vessels within the tumor tissue. In ovarian cancer, our group has identified the Type I EOC cells as the specific neoplastic cell population, which actively secrete these factors. Indeed, we previously showed that Type I, but not Type II EOC cells, secrete high levels IL-8, GROα, and MCP-1 [9].

Our new data however, show that Type I EOC cells, in addition to promoting a microenvironment conducive to vessel formation, play a more active role in the process of neovascularization (Fig. 7). In this report, we provide evidence showing that Type I EOC cells can serve as EPCs. To date, the most described EPCs are those derived from the bone marrow. However, unlike the Type I EOC population that we described in this study, bone marrow-derived EPCs are characterized as being $CD133^+$ and $CD34^+$ even prior to differentiation. None of these markers are found in the Type I EOC cells.

CD44 is a commonly used marker to enrich for CSCs. CD44 is a known signaling molecule and has been described to be the receptor for the extra-cellular matrix component, hyaluronic acid (HA). Interestingly, it has been recently reported that in atherosclerosis, HA, through CD44, may regulate intraplaque angiogenesis [26]. CD44 has also been described as part of the signaling cascade for macrophage inhibitory factor (MIF). Recently, it was reported that in addition to controlling cell cycle in brain tumors, MIF is also an essential factor in angiogenesis. Whether CD44 has a functional role on the capacity of Type I EOC cells to form vessel-like structures remains to be elucidated.

Recently, Shen et al. reported that precancerous stem cells (pCSCs) can serve as vascular progenitors [27]. They showed that xenografts from Green Fluorescent Protein-pCSCs contained blood vessels lined with GFP+ cells. However, analysis of pCSCs grown in hypoxic conditions only showed slight upregulation of CD31 and other angiogenic factors. Still, another group described a subset of $CD133^+$ ovarian cancer cells without tumor initiating properties as a cell population that can serve as vascular precursors through a VEGF-dependent process [28]. Taken together with our data, these findings suggest that there may be several phenotypes of cancer cells that can serve as vascular precursors. More importantly, the process that regulates differentiation of these distinct cancer cell populations may be diverse. Whereas VEGF is required for endothelial cell differentiation by non-stem $CD133^+$ ovarian cancer cells [28], IKKβ is required for the differentiation of Type I EOC cells with stem-like properties. These data highlight the possible diversity of differentiation stages among cancer cell populations.

Most anti-angiogenic therapies target the VEGF pathway. The humanized anti-VEGF compound Bevacizumab is currently in Phase III clinical trial for ovarian cancer. Results from pre-clinical studies showed that Bevacizumab can prolong survival in an in vivo ovarian cancer model [29]. However, results from recent clinical trials suggest that after an initial response to treatment, the anti-angiogenic effect of Bevacizumab is lost and neovascularization ensues. These new blood vessels are different from normal blood vessels; being more leaky and lacking a complete muscular layer. It is plausible that the formation of these vessels are VEGF-independent and therefore Bevacizumab is ineffective to prevent it. Considering our findings that the Type I EOC cell differentiation towards endothelial cells does not require VEGF, we postulate that these leaky blood vessels may originate from the Type I EOC cells. Taken together with our data, this suggests that additional compound(s), such as IKKβ inhibitors, which target the capacity of Type I EOC cells to differentiate, may be required to completely prevent the neovascularization process in ovarian cancer.

Summary

In summary, we demonstrate the capacity of Type I EOC cells to acquire an endothelial cell phenotype. This suggests that anti-angiogenic therapies should consider this inherent capacity of Type I EOC cells to serve as vascular progenitors.

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