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A Novel Population of Local Pericyte Precursor Cells in Tumor Stroma that Require Notch Signaling for Differentiation

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Commentary

Pericytes are a type of perivascular cell associated with the microvasculature which as other mural cells (e.g. vascular smooth muscle cells (VSMC)) are needed to support the endothelium and control vascular tone [1]. In tumors, pericytes aberrantly invest the microvascular bed [2]. Indeed, pericytes are commonly deficient and loosely associated with blood vessel capillaries [2], observations that have been frequently linked to increase leakiness of blood vessels, metastasis and thus worse cancer prognosis [3-5]. Recent clinical and preclinical animal model studies support the view that improved vascular wall pericyte investment normalizes blood flow which improves drug delivery and inflammatory cell infiltration and decreases metastasis [3]. Therefore, a better understanding of pericyte function could lead to therapeutic strategies to normalize tumor vasculature.

A major difficulty in studying pericytes is the absence of a specific method for their identification. Morphologic criteria, such as apposition with endothelial cells in microvessels are the most reliable current criteria to define pericytes [1]. However, this is not a reliable criterion in remodeling vasculature, especially in tumor where investment of pericytes is aberrant, e.g. loosely associated with the vascular wall [2]. In addition, this requires high-definition imaging, which does not allow for functional studies. Markers such as α-SMA, NG2, Desmin and PDGFRβ are often used to identify pericytes, although none of these individually is a bona fide marker that distinguishes pericytes from other mesenchymal cells such as the VSMC and fibroblasts, as both can have a perivascular location and express pericyte markers. In addition, the labile expression of these markers which varies during the differentiation of pericytes further hinders their identification. Therefore, it is crucial to develop new approaches to identify and isolate pericytes from tumors. CD146 (MCAM) has long been used as an endothelial marker, but a recent study demonstrated that a subset of $CD146^+$ cells that are also $CD45^-$ and $CD34^-$ identify pericytes from various normal mouse and human tissues [6]. We have recently developed a similar FACS based strategy substituting CD34 by CD31 to identify and isolate mouse tumor pericytes defined as $CD146^+/CD45^-/CD31^-$/lo cells. To ascertain that we sorted bona fide pericytes of stromal origin and not cancer cells with a similar marker signature, we implanted tumor models (LLC and B16F10) into transgenic syngeneic mice ubiquitously expressing GFP or CFP. In addition, for some experiments we implanted LLC-YFP tumor cells in transgenic GFP or CFP mice to exclude engulfment events (YFP^+ GFP^+CFP^+ cells) leading to misinterpretation of stromal identity. We validated by RT-qPCR for endothelial cell, pericyte and fibroblast markers the nature of the different GFP^+ or $CFP^+/CD45^-$ sorted populations by FACS relative to the expression of CD146 and CD31. In addition, we confirmed by immunostaining that a large fraction (80%) of $CD146^+/CD45^-/CD31^-$/lo stromal cells stain for the desmin pericyte marker, compared to the other sorted tumor stromal cell fractions e.g. endothelial cells $CD146^+/CD45^-$/ $CD31^+$ or pericyte precursors located in the $CD146^-/CD45^-/CD31^-$ compartment of the tumor stroma (described below).

We next used this FACS strategy to interrogate the source of pericytes in tumors. As bone marrow has been reported to be a source of pericytes [5-8] we first analyzed the proportion of tumor pericytes that were marrow-derived by quantifying $CD146^+/CD31^-$/lo cells in LLC and B16F10 tumors grown in GFP-tagged bone marrow transplanted animals. For this analysis we did not exclude $CD45^+$ cells as we did not want to exclude potential hematopoietic derived pericytes. We found that bone marrow contributes minimally to pericytes which prompted us to investigate another source for these cells. We hypothesized that a non-marrow precursor that resides in the tumor microenvironment differentiates into pericytes and that CD146 expression accompanies this differentiation. We thus examined the third non-tumor population of the tumor stroma, i.e. the non-pericyte, non-endothelial $CD146^-/CD45^-/CD31^-$ fraction to determine whether a pericyte precursor exists within this population. Indeed, we found that these stromal cells sorted by FACS from LLC and B16F10 tumors after 7 days of culture expressed the pericyte NG2 marker compared to freshly sorted uncultured cells. Characteristic to pericyte precursor cells [8,9] the presence of endothelial cells in co-culture further increased the differentiation of $CD146^-/CD45^-/CD31^-$ cells into $NG2^+$cells. In contrast, $CD146^-/CD45^-/CD31^-$ cells do not differentiate into endothelial, hematopoietic or fibroblast cells in culture as revealed by CD31, CD45 or FSP-1 immunostaining.

We then studied signalling regulating differentiation of $CD146^-/CD45^-/CD31^-$ cells into pericytes. We focused on Notch signaling as it is essential for many process of cell differentiation and notably for activating maturation of mural cells and their recruitment to blood vessels [10]. The components of Notch signalling comprise Notch receptors (Notch 1-4) and five membrane-bound ligands (Jagged1, Jagged2, Dll1, Dll3 and Dll4) whose roles have been extensively studied in normal and tumor endothelial cells [11] but remain poorly understood in tumor pericytes. Using cell sorting and RT-PCR analysis we first study the specific expression of the different Notch receptor and downstream Notch activation target genes of the Hey/Hes family. Notch 3 receptor and its target activation gene Heyl were found robustly increased in pericytes relative to EC and other sorted cell populations, an observation that concurs with previous reports studying the differentiation of other vascular mural cells such as VSMC [12]. Furthermore, we showed that coculture of endothelial cells engineered to overexpressed Jagged1 stimulate $CD146^-/CD45^-/$

$CD31^-$ cells to express the pericyte marker $NG2^+$, reminiscent of earlier studies sustaining the importance of the Jagged-1-Notch3-Heyl axis for perivascular mural cell differentiation and maintenance [12-14]. Conversely, drug-mediated inhibition of Notch signaling with a γ-secretase inhibitor decreased their differentiation into $NG2^+$ cells.

As we recently identified a $Tie1^+$/CD31dim/VE-$cadherin^-$/$CD45^-$ local precursor for VSMC during embryonic and adult arteriogenesis and differentiated by Notch [15], we hypothesized that Tie1 precursor cells are a source of tumor pericytes. To assess this we use a bitransgenic Tet-inducible animal to inhibit Notch signaling by expressing a dominant negative of mastermind (dnMAML-GFP) specifically in $Tie1^+$ cells [16]. In this system, we did not find that pericytes are derived from Tie1 expressing cells (based on dnMAML-GFP labelled cells) and neither does Notch inhibition in Tie1-expressing cells affect the abundance of pericyte cells in tumor, These findings led us to conclude that $CD146^-$/$CD45^-$/$CD31^-$ have a different origin from VSMC.

Based on previous observations that tumor pericyte arise from local immature mesenchymal cell recruited from the local tumor environment [17,18], we further extended the characterization of $CD146^-$/$CD45^-$/$CD31^-$ cells and showed that these cells expressed high level of the stem cell marker Sca1 and have increased expression of mesenchymal stem cell (MSC) markers CD13, CD44, Nt5e and Thy-1. However, we did not observe enrichment for Tie2 expression in $CD146^-$/$CD45^-$/$CD31^-$ cells, suggesting that pericyte precursors described herein are different than previously reported Tie2 pericyte precursors [18].

Conclusions and Perspectives

Our studies further extend our understanding of the source of tumor pericytes (Figure 1). We have defined a new method to identify and isolate pericyte from tumors. We determined that most pericytes are not derived from bone marrow, and we identified a new source of pericytes from the local tumor microenvironment, Sca1hi/ $CD146^-$/$CD45^-$/$CD31^-$ cells. Additional observations will be needed to further delineate the phenotype and origin of Sca1hi/$CD146^-$/$CD45^-$/$CD31^-$ cells. Interestingly, skeletal myoblasts in mouse embryo convert to pericytes when stimulated by the Notch ligand Dll4 and PDGF-BB [19]. Whether these muscle progenitor cells or other cells from the local tumor environment exists in normal adult tissues and contributes to tumor pericytes remains to be determined.

We also found that pericytes and their precursor Sca1hi/ $CD146^-$/$CD45^-$/$CD31^-$ cells differentially express Notch receptors and Notch activating genes of the Hey/Hes family and that Notch signalling is important for differentiation of precursors into pericytes based on our functional studies in vitro. Although, future studies will be important to further decipher the specificity of Notch receptors and ligands essential for this differentiation, our data support a role for Jagged1 in this context. Moreover, other pathways are possibly important for the differentiation of Sca1hi/ $CD146^-$/$CD45^-$/$CD31^-$ cells into pericytes such as TGF-β which cross-talks with Notch signalling for endothelial-pericyte heterotypic interactions through N-cadherin junctions [20]. Further, pericyte recruitment to the endothelium is mediated by multiple ligand receptor pairings, such as PDGF-B/PDGFRb, SDF-1a/CXC4R, HB-EGF/ErbB, Shh/Ptc, and Ang1/Tie-2 in the context of embryonic vascular development as reviewed elsewhere [1]. The challenge now will be to understand how these signalling pathways are involved in tumor vascular development in the context of cancer heterogeneity, and the aberrant pericyte and precursor populations present in tumors. Indeed, the answer is likely to be complex based on available data with PDGF-B mutant mice, which exhibit perycite recruitment and tumor growth responses differing among the tumor models tested [21,22].

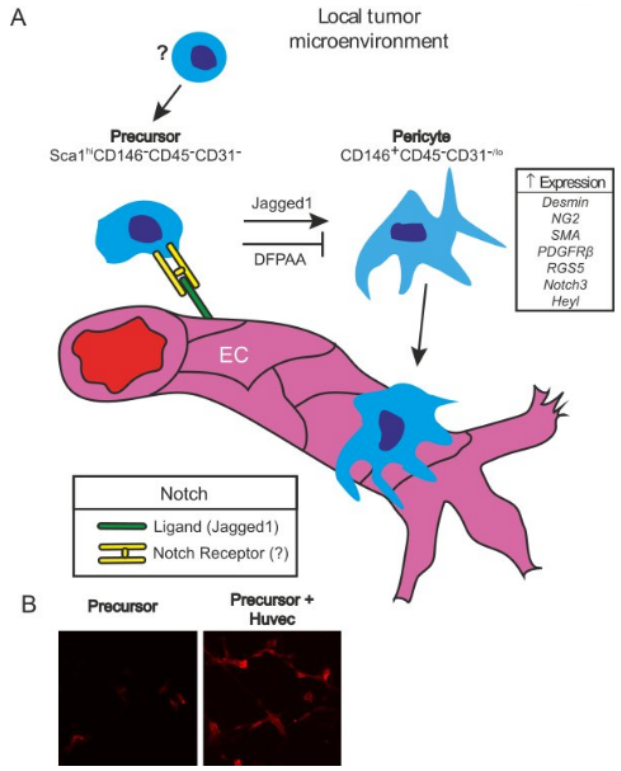

Figure 1: Findings summarizing the identification of pericyte precursors from the local tumor environment that differentiate in response to Notch signalling. A. Precursor and mature pericytes have distinct marker signatures that could be used for FACS isolation on the basis of CD146, CD31 and CD45 expression. Pericytes ($CD146^+CD45^-CD31^{-/lo}$) have high Desmin, SMA, NG2, PDGFRβ and RGS-5 gene expression in contrast to pericyte precursor cells (Sca1hi/$CD146^-$/$CD45^-$/$CD31^-$). The Notch 3 receptor and the Notch activation target gene Heyl are also robustly expressed in pericytes. The Notch ligand Jagged1 is expressed on endothelial cells (EC) and increases precursor differentiation into pericytes, while the Notch inhibitor DFPAA decreases it. The identification of Notch receptors important for precursor differentiation remains to be confirmed. The cell which pericyte precursors derive from is unknown. B. Fluorescent micrographs of precursor cells cultured in the absence or presence of EC (Huvec) for 7 days and stained for the NG2 pericyte marker. In the presence of EC the precursor show increased expression of NG2 and pericyte differentiated characteristics such as extend cytoplasmic processes.

What are the functions of pericytes and their precursors Sca1hi/ $CD146^-$/$CD45^-$/$CD31^-$ cells for cancer progression? One motivation to study pericytes and their sources stems from the hypothesis that increasing pericyte investment of the tumor vasculature could normalize blood flow and thus improve anticancer drug delivery and

immune cell infiltration, reduce metastasis and ultimately improve cancer outcomes. This concept was validated previously in mice deficient for RGS5, a G protein regulator that inhibits the maturation of pericytes that is strongly expressed in different cancers [23]. However, a recent study in human and mouse ovarian tumors suggested that high pericyte content predicts poor patient prognosis and promotes malignancy in ovarian cancer independent of angiogenesis [24]. The mechanism behind this cancer promoting role of pericytes remains unknown at present. Hypothetically, pericytes (and possibly their precursors such as Sca1hi/CD146^{-}/CD45^{-}/CD31^{-} cells) could be a source of other cell types in tumor that are potential driver of tumor progression, such as cancer associated fibroblasts. In support of this hypothesis, previous studies have pointed out the stem cell like characteristics of pericytes in normal tissues for the generation of multiple mesenchymal cell lineages [6]. Whether this stem-like function of pericytes to generate fibroblasts in pathologic fibrosis remains controversial [1,25], and has not yet been reported in cancer. Nevertheless, these studies highlight the potentially complex roles of pericytes in cancer and thus the need to further understand the specific functions of these cells and their sources in cancer heterogeneity. In this context, our findings will be helpful to unravel these functions.

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