Endothelial Cells Form a Phalanx to Block Tumor Metastasis

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Tumor blood vessels deliver oxygen poorly, thereby contributing to tumor hypoxia and upregulation of proangiogenic cytokines in an escalating feedback loop. Mazzone et al. (2009) now show that reducing the amount of a protein involved in endothelial oxygen sensing leads to changes in endothelial cell shape that interrupt this feedback loop and reduce tumor metastasis.

There is a paradox in tumor biology that only became obvious when antiangiogenic drugs became available for testing in animal models and clinical trials. Most tumors require their own blood supply to grow, and once a tumor has made the "angiogenic switch," it is capable of inducing blood vessel formation to supply its metabolic needs (Hanahan and Folkman, 1996). Although it was thought that compounds that block tumor angiogenesis would be effective in cancer therapy, to date the available drugs only transiently block tumor growth. There are likely numerous reasons for this outcome (Bergers and Hanahan, 2008), but one interesting possibility centers around the peculiar characteristics of tumor-induced blood vessels. Tumor blood vessels are leaky and inefficient at oxygen delivery. As a result, the tumor remains oxygen starved, or hypoxic. Hypoxia stimulates tumor cells to continue production of cytokines and growth factors, including proangiogenic factors that over-stimulate tumor blood vessels and keep them dysfunctional. It has been proposed that "normalizing" blood vessels to better perfuse the tumor tissue may actually downregulate the signaling pathways that contribute to tumor growth (Jain, 2005). In this issue, Mazzone et al. (2009) show that perturbing the oxygen-sensing pathways of endothelial cells leads to normalization of tumor blood vessels. Moreover, this endothelial normalization dramatically blocks tumor invasion and metastasis, suggesting that endothelial oxygen sensing may be a compelling target for cancer therapy.

The study focuses on a component of cellular oxygen sensing called prolyl hyroxylase domain protein 2 (PHD2). PHD2 is one of a group of proteins that hydroxylate critical residues in subunits of hypoxia-inducible factors (HIFs), a modification that targets the HIFs for degradation (Semenza, 2003). When oxygen tension is normal, HIF degradation prevents the activation of downstream targets in the hypoxic response. To activate the hypoxic response, PHD activity is downregulated and HIF subunits are stabilized. Mazzone et al. genetically deleted *PHD2* and found that complete loss of *PHD2* is embryonic lethal, consistent with previous findings (see for example Takeda et al., 2006). Thus, the authors focused on the effects of heterozygosity for *PHD2* (*PHD2*$^{+/-}$). *PHD2*$^{+/-}$ mice express *PHD2* at 50% of wild-type levels and have normal blood vessel parameters under physiological conditions. They then used xenografts to produce wild-type tumors that contain blood vessels with reduced amounts of PHD2. They hypothesized that reduction of endothelial PHD2 via heterozygosity would lead to increased tumor invasion and metastasis due to hypoxia-driven upregulation of signaling pathways. Instead, they found the opposite result—tumors with blood vessels that expressed less PHD2 were better perfused, less invasive, and much less metastatic than the same tumors with wild-type blood vessels.

The authors go on to show that tumor blood vessels with reduced PHD2 are as abundant as wild-type tumor blood vessels, but the blood vessels expressing less PHD2 resemble normal, non-tumor blood vessels. They are less leaky, have better pericyte coverage, and have a more consistent basement membrane—all hallmarks of mature, quiescent vessels. However, the most salient differences between wild-type and PHD2-reduced tumor blood vessels pertain to their physical appearance and expression profile. Scanning electron micrographs of the inner blood vessel wall showed that endothelial cells of wild-type tumor blood vessels were disorganized, with cells inside the lumen and gaps between the cells. In contrast, the endothelial cells of blood vessels with reduced PHD2 had a uniform shape that produced an even and smooth lining with no gaps. The authors termed this uniformity a "phalanx" phenotype (a phalanx is a seamless formation of soldiers along a battle line, in military parlance). Conditional heterozygosity for *PHD2* in endothelial cells recapitulates these effects, indicating that vessel normalization is initiated by endothelial cells.

How might changes in a cell's oxygen-sensing machinery set into motion events leading to vessel quiescence and normalization? The gene expression profile of freshly isolated endothelial cells shows that both isoforms of the vascular endothelial growth factor receptor Flt1

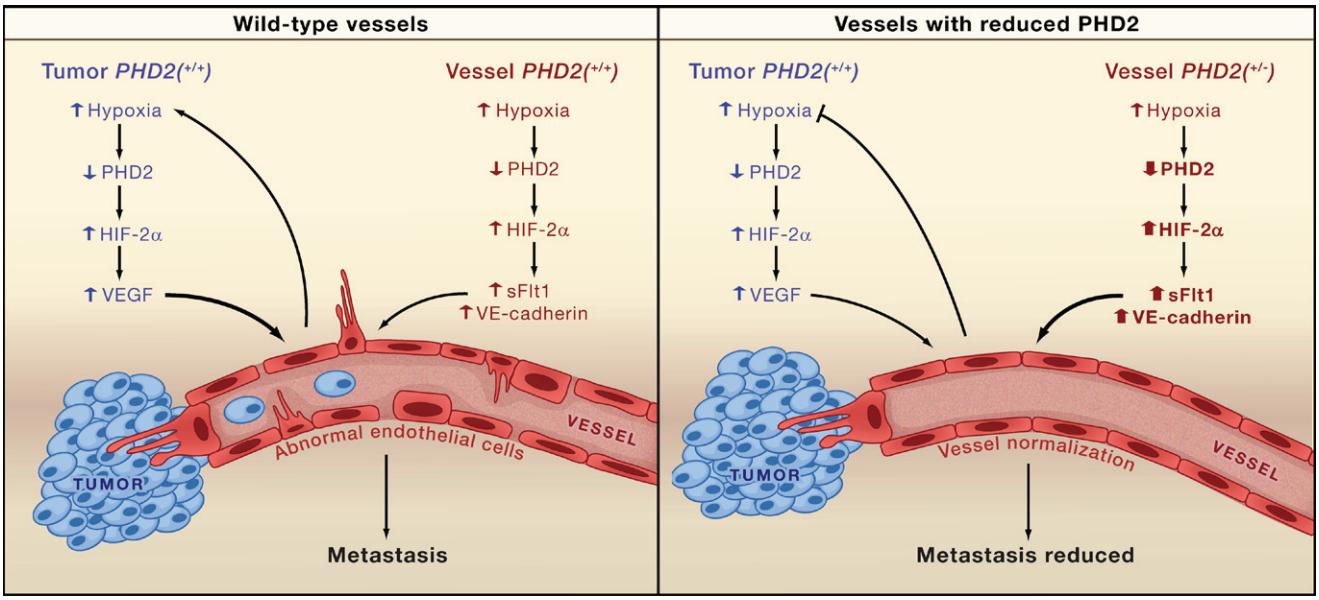

Figure 1. Reduction of PHD2 Inhibits Metastasis

Tumor environments are hypoxic, which leads to upregulation of cytokines such as vascular endothelial growth factor (VEGF) that over-stimulate entering blood vessels. In endothelial cells, downregulation of prolyl hydroxylase domain proteins (PHDs) leads to upregulation of hypoxia-inducible factors (HIFs). However, the stimulation of downstream effectors of HIFs, including vascular endothelial cadherin (VE-cadherin) and soluble Flt1 (sFlt1), is insufficient to overcome cytokine stimulation. Blood vessels are leaky, open to invasive metastatic tumor cells, and deliver oxygen poorly. This leads to escalating tumor hypoxia. Tumors vascularized with blood vessels that express reduced PHD2, however, are less hypoxic and invasive. This results from a normalization of blood vessel morphology, in which endothelial cells adopt a "phalanx" phenotype characterized by smooth nonleaky blood vessels that effectively deliver oxygen and block metastasis. Increased expression of sFlt1 (a negative regulator of VEGF signaling) and VE-cadherin (which stabilizes junctions) are thought to contribute to the quiescent, normalized endothelial phenotype.

(VEGFR-1) and the junction protein vascular endothelial-cadherin (VE-cadherin) are markedly upregulated. This finding suggested that these proteins could be downstream effectors of oxygen-dependent vessel normalization. The soluble Flt1 (sFlt1) isoform is a negative regulator of VEGF signaling (Kendall and Thomas, 1993), and VE-cadherin is found in junctions but is also a critical regulatory node for junction assembly and endothelial barrier function (Taddei et al., 2008). The link to oxygen sensing is strengthened by experiments showing that HIF-2α regulates expression of both potential effectors. Indeed, in cell culture, knockdown of HIF-2α is more effective than knockdown of HIF-1α in reducing expression of both VE-cadherin and sFlt1. Moreover, HIF-2α protein is localized to tumor vessels. Other groups have shown that both VE-cadherin and Flt1 have hypoxia-response elements in their promoters that are recognized by HIF-2α (Dutta et al., 2008; Le Bras et al., 2007).

Thus, a model emerges for how the hypoxic response may differentially affect tumor cells and endothelial cells and how this might lead to the surprising finding that reduction of PHD2 activity in endothelial cells blocks tumor invasion and metastasis (Figure 1). In a hypoxic tumor environment, entering blood vessels downregulate PHDs and upregulate HIFs. In endothelial cells, HIF-2α is sensitive to PHD2 levels, and a reduction of PHD2 to half of normal levels allows downstream effectors of HIF-2α, such as sFlt1 and VE-cadherin, to overcome the influx of tumor-derived hypoxia signals that induce dysfunctional blood vessels. The PHD-deficient blood vessels form a "phalanx" phenotype that improves oxygen delivery, reduces tumor hypoxia, and blocks intravasation of invasive and metastatic tumor cells into the blood vessels. In contrast, wild-type blood vessels have more PHD2 and less HIF-2α, VE-cadherin, and sFlt1. Thus, they cannot overcome the influx of tumor signals and remain leaky and open to invasive tumor cells. Moreover, these wild-type blood vessels do not improve oxygen delivery and so cannot break the cycle of signaling induced by the hypoxic response of tumor cells. This may lead to an escalating feedback loop.

There are numerous interesting questions provoked by these findings and this model. It will be important to show that these findings are relevant in spontaneous tumors that closely mimic tumors seen in the clinic. It will also be exciting to see how other signaling pathways that affect angiogenesis and vessel morphology intersect with this regulatory module. For example, platelet-derived growth factor (PDGF) signaling and signaling of angiopoietin through Tie receptors can also promote vessel quiescence. The role of other components of the oxygen-sensing pathway in blood vessel normalization requires further clarification, especially given that HIF-1α regulates a gene set that does not completely overlap with the set regulated by HIF-2α.

The work of Mazzone et al. is significant in several ways. The data highlight that the endothelial cell's ability to sense oxygen has an important impact on blood vessel morphogenesis and function. The description of the "phalanx," a new endothelial phenotype that is oxygen sensitive, underscores

the notion that phenotypic heterogeneity of endothelial cells within blood vessels is critical to proper development and function. The work provides an example of how changes to cell shape rather than cell numbers dramatically affects blood vessel phenotype and tumor progression. Finally, blood vessel normalization affects tumor invasion and metastasis, suggesting that endothelial oxygen sensing may represent a new target of cancer therapy.

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In DNA Replication, the Early Bird Catches the Worm

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The initiation of DNA replication is a complex, multistep process with important implications for genomic stability. In this issue, Wu and Nurse (2009) find that initiation factors are differentially recruited to replication origins. They uncover evidence suggesting that the efficiency of this recruitment may determine whether and when an origin is used to initiate DNA replication in S phase.

Most cells spend a large portion of the cell cycle preparing to replicate their DNA. Replication initiates from regions in the chromosomal DNA called replication origins in a process referred to as origin firing. These origins attract the six origin recognition complex (ORC) proteins during mitosis. The binding of ORC proteins to the origin serves to initiate a multistaged process involving the recruitment of a series of different proteins that results in the separation of the two DNA strands, loading of the DNA polymerase, and initiation of DNA replication (Figure 1). Yet, virtually nothing is known about the mechanisms by which these regions, crucial to replication, are selected and when this selection is made. Furthermore, unlike in prokaryotes, in eukaryotes there are no known characteristics that can help to predict whether a given DNA sequence can serve as an origin of DNA replication. A single mammalian cell harbors tens of thousands of potential replication origins, yet only a subset of these is used in each particular S phase. Thus, the activities of these origins must be strictly regulated. Indeed, defective control of DNA replication can lead to mutations, genomic instability, and cancer. In this issue of *Cell*, Wu and Nurse (2009) find evidence suggesting that in the fission yeast, *Schizosaccharomyces pombe*, the ability of an origin to attract ORC proteins during mitosis determines to a large extent the timing of initiation factor assembly at the origin and whether that origin is selected as a replication initiation site in the following S phase.

To examine how replication origins are selected, Wu and Nurse measured the kinetics with which initiation factors, including ORC proteins, bind to origins in fission yeast. The authors found that origins known to be efficient ("good" origins used in almost every S phase) bind to ORC proteins earlier and with greater affinity than origins that are not frequently used ("poor" origins). This correlation could also be seen in the efficiency with which prereplicative complexes (pre-RCs) and pre-initiation complexes (pre-ICs) are formed at different origins. Furthermore, if the time available for ORC proteins to bind at origins is increased by adding a drug that prolongs mitosis, Wu and Nurse observed that efficient origins become less efficient, whereas poor origins are used more frequently. These experiments substantiate the claim that the pattern of ORC protein binding determines which potential origins are actually used in the ensuing S phase. It is possible that increasing the length of mitosis allows ORC protein binding to become equalized between