# Tumor vasculature address book: Identification of stage-specific tumor vessel zip codes by phage display

**Using in vivo phage display technology with murine tumorigenesis models, two reports have identified peptide motifs that selectively home to distinct molecular vascular targets in a tumor type- and stage-specific manner (Hoffman et al., 2003 and Joyce et al., 2003 [this issue of *Cancer Cell*]). By probing the surface-protein repertoire of these unique vascular beds, a pioneering draft of a molecular roadmap to decipher the heterogeneity of the vascular system in the context of carcinogenic progression has been plotted. Analysis of these phage peptides that differentially home to dysplastic or invasive tumor vasculature will lay the foundation for identifying unique functional tumor vascular-specific motifs that could potentially be applied to targeted therapeutic and imaging modalities.**

Several lines of evidence have shown that the vasculature of each organ expresses a unique set of functional molecules that dictate endothelial heterogeneity specific to that particular organ. Similarly, tumor vasculature expresses unique markers that distinguish it from normal vasculature. The question of whether tumor vasculature during carcinogenic progression has stage-specific molecular changes has not been addressed. This is primarily due to a lack of models or technology to identify such transiently expressed surface proteins during tumor progression. Two reports in this issue of *Cancer Cell* have identified unique molecular signatures that are specifically expressed at different stages of tumor progression from a highly proliferative and angiogenic dysplastic lesion to invasive phase by combining phage display techniques with transgenic mice engineered to develop spontaneous tumors. They have identified a set of phage peptide motifs that specifically home to the vasculature of either dysplastic or invasive pancreatic and squamous skin cancers. These studies will most likely lay the foundation for identifying unique therapeutic targets that are selectively expressed on the tumor vasculature.

What is unique about tumor vasculature that provides a distinct platform for selective phage peptide homing? Tumor vessels are structurally and physiologically different than normal vasculature (Carmeliet and Jain, 2000; Hanahan and Folkman, 1996). Typical tumor vessels are tortuous, leaky, and discontinuous, and they are invested with disorganized peri-endothelial supportive cells. The determinant of tumor vasculature heterogeneity is dictated in part by dysregulated expression of tumor-derived angiogenic factors, including VEGFs, FGFs, PDGFs, as well as yet unrecognized angiogenic factors. Overexpression of the angiogenic factor, VEGF, results in generation of unstable, dilated, leaky neovessels and induction of expression of adhesion molecules promoting infiltration of inflammatory cells (Benjamin et al., 1999; Bergers et al., 1999). Moreover, recruitment of endothelial cells (ECs) from local preexisting vasculature as well as circulating marrow-derived endothelial progenitors may provide for another dimension of tumor vasculature heterogeneity (Rafii et al., 2002).

Identification of tumor stage-specific endothelial markers has been hindered in part by technical difficulties in isolating pure population of functionally intact and phenotypically stable populations of ECs from various carcinogenic stages. In addition, in vitro cultivation of purified populations of ECs may lose their enduring stage-specific traits. To circumvent these problems, several groups have used highly sophisticated molecular and immunological approaches to identify unique markers that are expressed on the surface of tumor but not on normal vasculature. Serial analysis of gene expression (SAGE) is a mRNA profiling technology in which clones of concatenated short sequence tags (10–17 bp) derived from mRNA of targeted cells are generated, sequenced, and analyzed with a deconvolution algorithm (St. Croix et al., 2000). The major strength of the SAGE technique is its ability to quantify global gene expression without prior sequence information. SAGE has been used to identify genes and ESTs that are selectively expressed or upregulated in the human colon cancer relative to normal vasculature (St. Croix et al., 2000). However, genes identified by SAGE analysis require additional time-consuming and cumbersome development of reagents to validate their tumor vasculature specificity and functionality.

An alternative and complimentary means to probe tumor vasculature that allows for rapid identification of specific markers involves in vivo phage display technology. Phage display peptide libraries contain peptide motifs that can home to the tumor vasculature and bind directly to the molecules expressed on tumor vessels (Arap et al., 1998; Pasqualini and Ruoslahti, 1996; Ruoslahti, 2002). In vivo peptide phage display screening technology has the added advantage of identifying stage-specific, functionally active, surface-expressed proteins of the tumor vasculature. In some circumstances, peptides expressed on the phage may also block the function of the proteins, thereby providing critical information about the angiogenic properties of the targeted proteins.

Identification of unique molecular signatures that are selectively expressed in each stage of tumor vasculature from dormant/dysplastic to invasive phase has several important therapeutic and diagnostic applications. Peptides that target the premalignant dysplastic phase of tumor vessels can be used diagnostically to localize early tumors and possibly be used therapeutically in a neoadjuvant or adjuvant setting to prevent the angiogenic switch (Figure 1). Functional blockade by such peptides that home to invasive tumors will also allow radiological localization of the micrometastatic tumors and can be used as antiangiogenic agent.

Screening by phage peptide libraries can be performed either in vitro or in vivo. Phage display libraries contain an average collection of bacteriophage particles displaying random phage peptides in as many as $10^9$ permutations. By exposing the vasculature to phage libraries by systemic perfusion and/or to isolated primary cells ex vivo, peptides that selectively bind can be enriched through repetitive cycles of binding and amplification. This will allow for rapid

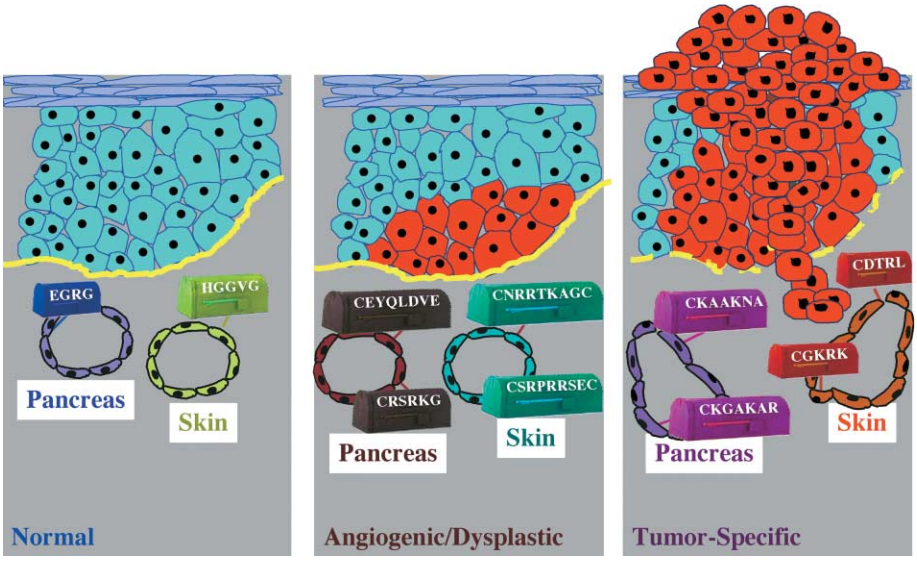


**Figure 1.** Homing of phage peptides to distinct "molecular zip codes" on endothelial cell surface

Selective binding of these peptides correlates with the progression of tumor in a stage-specific manner. The homing peptides EGRG and HGGVG differentially bind to normal pancreas and skin vasculature, respectively. In the K14-HPV16 squamous tumorigenesis model, phage peptides CNRRTKAGC and CSRPRRSEC are specific for dysplastic skin neovasculature while CDTRL and CGKRK are selective for tumor neovasculature. In the RIP-Tag tumorigenesis model, phage peptides CRSRKG and CEYQLDVE target for angiogenic/dysplastic pancreatic islets while CKAAKNA and CKGAKAR home to pancreatic tumor neovasculature.

identification of genes that are upregulated from normal to tumor blood vessels. A number of published studies with phage libraries have reported the identification of vascular targeting receptors in tumors, primarily in mice (Giordano et al., 2001; Pasqualini and Ruoslahti, 1996; Ruoslahti, 2002) and also in a patient (Arap et al., 2002b). In vivo screening of phage libraries in murine models has identified specific motifs, including RGD, GSL, and NGR, that bind to integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$, matrix metalloproteinases, and vascular growth factor receptors that are upregulated in neoangiogenic tumor ECs (Pasqualini and Arap, 2002; Pasqualini et al., 2002; Ruoslahti, 2002). Using this technology, IL11 receptor has been identified to be selectively expressed in prostate vessels (Arap et al., 2002a; Pasqualini and Arap, 2002), and the aminopeptidase P has been shown to target breast tumor vasculature (Essler and Ruoslahti, 2002). However, these studies did not provide a temporal pattern of selective phage homing during tumor progression from premalignant to the invasive phase, and homing of RGD, GSL, and NGR peptides was independent of the origin of the tumor.

In the current report by Hoffman et al. (2003), a heptameric cyclic phage ($CX_7C$) peptide library was used to identify homing peptides that selectively bind to the dysplastic and squamous carcinomas of K14-HPV16 transgenic mice. One peptide that selectively homed to the tumor endothelial cells was identical to a sequence in the human kallikrein-9 protease. In the companion report using a combined in vitro and in vivo phage library, Joyce et al. (2003) demonstrate that there exists specific surface protein differences in the vasculature of pancreatic tumors in a RIP-Tag2 transgenic mouse model of tumorigenesis. By employing the aforementioned peptide phage panning techniques, they probed cells with access to the vasculature, in particular, ECs, pericytes, and tumor cells. Peptide motifs specific to normal, premalignant and tumor vessels that imply that the vasculature has a distinct and specific progression in line with tumor development were obtained. One peptide homing to the tumor vessels was found homologous to pro-PDGF-B. Additionally, they detected differences in the peptide binding profile between the vasculature of a pancreatic islet tumor and an ectopic implanted tumor, which suggests a context-dependant molecular signature for tumors. The finding that pro-PDGF-B peptide was only detected in the pancreatic tumors may be due to the finding that as compared to skin squamous cancers, RIP-Tag2 pancreatic tumors are leaky and develop micromorrhages, therefore exposing these peptides to PDGF receptor-positive pericytic cells. Nonetheless, tumor vascular leakiness is a unique feature of certain tumors that may be exploited to target specific peptides. By combining in vivo phage display panning techniques with a progressive genetic tumor model, these groups have been able to track the differential surface protein repertoire of tumor vasculature in its progression from dormant dysplasia to invasive metastatic disease.

However, these two studies also have few shortcomings. It is not clear whether kallikrein-9 protease or pro-PDGF-B play any significant physiological role during tumor progression or just serve as unique stage-specific vascular molecular signatures. Moreover, there are also the potential hurdles associated with the translation of the data derived from mouse models to humans. Particularly, stage-specific peptide probes isolated in mouse tumor models may not always be replicable in human tumors. Indeed, the expression pattern of molecules in human tumors may be drastically different from the RIP-Tag pancreatic or the HPV16 skin carcinogenesis models. In addition, the degree of tumor vascular leakiness and stabilization may also differ significantly in tumors developing in murine models of tumorigeneiss and de novo human cancers. Wound healing and organ regeneration after tissue ischemia requires formation of neovessels, which may express a similar surface protein profile to tumor vasculature. Therefore, it remains to be determined whether any of the stage-specific peptides will also home to the neoangiogenic vessels during tissue revascularization after ischemic damage. It is possible that a certain subset of peptide motifs may have selective activity for tumor vessels and that some peptides are inclusive of all newly formed vessels. Nonetheless, identification of these known and as of yet novel tumor vasculature-specific peptides will provide a platform for future studies to challenge the role of

these factors in the modulation of neoangiogenesis. In addition, it remains to be determined whether these peptides, such as dysplasia-specific homing peptide CSRPRRSEC, play any significant role in wound healing, tissue revascularization after ischemia, or whether this homing is a salient feature of tumors.

Taken together, these findings set forth an enticing hypothesis of a functional vascular heterogeneity that can be studied and used in the future to understand the underlying pathogenesis of tumor neoangiogenic processes. This approach will also provide for new molecular targets for radiographic tumor detection and molecularly directed chemotherapeutic drug delivery and drug sensitivity testing. Given the fidelity in homing of these peptides to their vascular targets, fusion of a specific peptide with an antiangiogenic agent will facilitate delivery of high concentrations of a toxin to disrupt tumor vessels. In light of the unparalleled specificity of phage peptides, there is no doubt that this technology will allow us to identify stage-specific tumor and organ-specific vascular zip codes. This will facilitate identification of therapeutic targets to block tumor angiogenesis and inhibit angiogenic switches with minimal toxicity to other tissue organs, such as those during postnatal development or physiologically regenerative processes.

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# Modeling transformation and metastasis in *Drosophila*

**Transformation and metastasis are complex, multistep processes. Two recent papers exploit powerful *Drosophila* genetics techniques to explore cooperation between multiple genetic manipulations and to model these processes. In particular, oncogenic Ras is found to collaborate with disruption of cell polarity to trigger massive neoplasia and metastasis. These studies promise further progress in research into the causes of cancer.**

Cancer has long been thought of as a multistep process, involving cooperation between several oncogenic events and mutations in tumor suppressors to yield full-blown cellular transformation and metastasis (Hanahan and Weinberg, 2000). *Drosophila* possesses ideal tools to study the clonal effects of multiple genetic manipulations on populations of cells alongside phenotypically normal cells. However, its potential for studying the stepwise nature of the transformation process had so far been largely untapped. This looks set to change with the publication of two complementary reports by the laboratories of Tian Xu and Helena Richardson focusing on cooperation between multiple genetic events to promote neoplastic growth and metastasis (Brumby and Richarson, 2003; Pagliarini and Xu, 2003).

These studies were facilitated by the use of the MARCM technique (Lee and Luo, 2001). Importantly, the MARCM technique allows the generation, in a heterozygous animal, of clones of cells that (1) are homozygous mutant for various mutations, (2) overexpress a transgene(s) of interest, and (3) are labeled positively with a GFP marker. Thus, the MARCM technique is a versatile tool with which the consequences of multiple genetic manipulations upon positively marked cells can be examined in vivo. In both studies, the formation of these clones was targeted to the eye imaginal disc.

### *A model for metastasis in* Drosophila

The Ras protooncogene is activated in about 30% of human cancers (Hanahan and Weinberg, 2000). Ras has been implicated in the metastatic process in mammalian systems, though its precise in vivo contribution is not fully under-