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Antiangiogenic Signaling Network Induced by Endostatin

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Angiogenic response is characterized by a large number of individual genetic signalings which are highly coordinated and interdependent. This could be the reason why a unified model to describe the multifaceted antiangiogenic effect of endostatin has not been reported. In this work the pan-genomic consequences of the administration of the endogenous inhibitor endostatin were examined, to determine how these signals are expressed collectively in response to a specific angiogenesis perturbation. We performed the largest genome array analysis to date to observe the entire orchestration of gene expressions of a single protein on a normal cell population. We demonstrate here for the first time that when treating normal tissue with an endogenous protein, gene arrays can reveal global network patterns which drive amplification of function. This is potentially useful for assessing homeostatic mechanisms in normal tissue biology and for understanding the system-wide genetic changes which accompany disease states. By not defining any genetic subset a priori (e.g. by use of a specialized angiogenesis chip), we were able to preserve the intact signaling network that defines the unabridged genomic influence of endostatin. This differs from a conventional gene array study in which cancer cell expressions are compared to normal cell expressions. This thesis takes advantage of gene profiling and protein phosphorylation studies to determine the global antiangiogenic effect of endostatin on human endothelial cells. In addition to array data the antibody arrays showed that for many of the pathways, the phosphorylation status of individual proteins was altered in similar manner after endostatin treatment. Eight signaling cascades that were down-regulated by endostatin treatment were investigated in more detail: Id and AP-1 signaling (cell proliferation), HIF-1 α signaling (low oxygen and metabolic adaptation), ephrin and TNF- α signaling (cell migration, tube formation), NF- κ B (proliferation and antiapoptosis), STAT signaling (regulator of proliferation migration and survival), Ets (migration and antiapoptosis), coagulation cascades, and adhesion molecule pathways. These results identify several new pathways regulated by endostatin and present a picture of a complex network of responses to a single ligand.

We have found that endostatin, a specific angiogenesis inhibitor, orchestrates a very extensive system of genes in endothelium to accomplish the single task of downregulating pathologic blood vessel growth. Surprisingly, what we observed in response to endostatin treatment is the coordinate activity of entire classes of genes rather than controls by specific gene players. This entirely reforms our notion of a molecular "chain of command" in the angiogenic process. Highlighted in this thesis is the unique finding that the set of gene expressions underlying the angiogenic balance in tissues can be molecularly reset en masse by a single

protein. Revealed is an unprecedented, pan-genomic alignment of angiogenic gene regulation (downregulation of angiogenic genes and upregulation of anti-angiogenic genes) that amplifies the anti-angiogenic action of endostatin. Not only is this of general interest in angiogenesis, oncology, and development, but these results expose a novel principle of self-defining gene classification. This new mechanism elucidates a unique role for arrays themselves that is relevant to network theory, bioinformatics and proteomics.

The take-home message of this study is threefold. Because our array provided a global view of genetic response following treatment with a single endogenous protein, in this case endostatin, it was able to show that: 1) pathways thought to be distinct show novel cross-connections; and 2) angiogenic genes as a class tend to be downregulated while anti-angiogenic genes tend to be upregulated; resulting in 3) a unique, genome-wide amplification of function that has implications for the understanding of genetic networks extending well beyond angiogenesis.

1) Evidence is presented for cross-talk among pathways assumed to be distinct. This study led to the absolutely unexpected finding that endostatin downregulates HIF-1 α , one of the most powerful hypoxic response factors, and simultaneously upregulates an antagonist of HIF-1 α (HIF-1AN). Because the HIF response may therefore not be expected under antiangiogenic therapy, this surprising finding counters the argument now being made by some scientists that a HIF-driven hypoxic response will be the Achilles' heel of anti-angiogenic therapy. This work also underscores how intertwined angiogenesis is with other biological disciplines. It is noted, for instance, that the Adenomatous Polyposis Coli (APC) tumor suppressor gene (a gene heretofore not associated with angiogenesis) is highly upregulated by endostatin, raising the possibility this suppressor activity may function in part through angiogenesis inhibition. The angiogenic response network is shown to be more complicated and extensive than previously appreciated.

2) Remarkably, angiogenic genes are aligned with downregulated genes while anti-angiogenic genes are aligned with upregulated genes. This pattern speaks to an evolutionarily entrenched role for endogenous endostatin in orchestrated vascular development and remodeling. A practical application of our results lies in the search for new angiogenic or anti-angiogenic genes.

3) Perhaps most impressive is the alignment of function seen for the regulated genes as a group. Because angiogenic genes are downregulated and anti-angiogenic genes are upregulated, the original function of endostatin appears amplified. This study revealed the unexpected finding that endostatin upregulates thrombospondin. Thrombospondin is a major endogenous angiogenesis inhibitor which is downregulated by ras during the switch by cancers to the angiogenic phenotype. The fact that endostatin upregulates thrombospondin and thus provides additional inhibition of endothelial response to tumors is especially instructive in that one protein upregulates another where both are carrying out the same function, i.e., inhibition of endothelial cell proliferation and migration. This is a novel form of amplification of function. It reveals processes that cannot be readily deduced from the detailing of genetic players. We believe the genome-wide amplification of function seen for endostatin is an unprecedented finding. This study clearly constitutes an unveiled "global pattern" useful for analyzing the biological condition of anti-angiogenic response which is new and unusually instructive in that it reveals the large extent of genetic cooperation that is potentially available to accomplish a single, defined function. Evoked is a concept of a 'super pathway' where a sizable fraction of the entire genome is cooperating to a specific end through an endostatin control 'hub'. Beyond angiogenesis, this has ramifications for any field in biology where elucidation of a genetic network or sub-network is an objective. These results will therefore be of interest to those in fields ranging from basic genetics to biochemistry to genetic network theory, bioinformatics and proteomics.

This study resolves some of the controversies surrounding endostatin's biology, and provides a new direction to help dissect the molecular pathways involved in endostatin's selective tumor antiangiogenic effects. It is here demonstrated for the first time that the set of gene expressions underlying the angiogenic balance in tissues can be molecularly reset en masse by a single protein. These results point to a pervasive and entrenched role for endostatin in the regulation of endothelium. Profiling further reveals the regulation of certain wellcharacterized genes not heretofore associated with angiogenesis. Together, these complex inter-pathway communications triggered by endostatin comprise an intricate signaling network activated in endothelium that both recapitulates and extends on current understanding of the angiogenic process.