Identification of IRX3 associated Transcription Factors ADORA2A, CYR61, DKK1, and EP300 during VEGF-Induced Endothelial Cell Angiogenesis

Nitish Sood, McKay Mullen, and Leonard M. Anderson, PhD
Cardiovascular Research Institute, Department of Physiology, Morehouse School of Medicine

Abstract

BACKGROUND: Excessive and insufficient angiogenesis can result in a plethora of different diseases. Excessive angiogenesis is associated with range of pathological conditions ranging from rheumatoid arthritis to cancer, through tumor neovascularization and eventually metastasis. Insufficient angiogenesis can exacerbate the damage caused by a stroke and even cause infertility. This is due to the prevention of proper tissue recovery after ischemic diseases. The proliferation of these conditions has contributed significantly to morbidity rates around the United States. Angiogenesis is a "common denominator" shared by diseases affecting more than one billion people worldwide. This includes all cancers, cardiovascular disease, blindness, arthritis, complications of AIDS, diabetes, Alzheimer's disease, and more than 70 other major health conditions affecting children and adults in developed and developing nations. Therapeutic intervention of pathological conditions associated with angiogenesis could reverse their effects and even be utilized as a preventative measure. During angiogenesis, endothelial cells lead to sprouting vessels, extend numerous dynamic filopodial extensions, and migrate according to chemical gradients. This results in the formation of new blood vessels. Vascular Endothelial Growth Factor A (VEGFA) creates a critical chemical gradient as a signal protein and triggers a signaling cascade, resulting in increased angiogenesis. Although some factors involving this process are known, current research falls short in defining the entire genetic regulatory network. Iroquois Homeobox Gene 3 (*Irx3*) is a transcription factor and member of the 'Three Amino Acid Loop Extension' (TALE) class of homeobox genes. Recent literature suggests that *Irx3* is up-regulated by VEGFA. Current known mediators which regulate angiogenesis include E26 transformation specific (ETS-1), Forkhead Box 'O' (FoxO), and ETS-Related Gene (ERG). ETS-1 regulates VEGF-Receptor 2 during hypoxic conditions stimulated by Hypoxia-Induced Factor 2 Alpha; FoxO reduces the speed of angiogenic effects post-exercise; ERG is essential in regulating the balance between apoptosis and survival in ECs as well as vascularization through the VE-cadherin-GFP receptor. There is no recent literature identifying co-factors *Irx3* binds with to regulate angiogenesis. Our research seeks to identify novel co-factors that *Irx3* binds with during VEGF-stimulated angiogenesis.

HYPOTHESIS: Our central hypothesis is that *Irx3* is binding with transcription targets which function as 'pro-' or anti-angiogenic mediators in response to VEGF in Endothelial Cells (ECs) .

RESULTS: Our results have located specific focus genes that *Irx3* binds with during VEGF-stimulated angiogenesis. These genes were identified through the utilization of bioinformatic analyses to cross-reference and filter results from the Chromatin-Immunoprecipitation assays with DNA Microarray (ChIP-on-Chip) experiment. The results of this experiment show that the area around the Transcriptional Start Site (TSS) had increased enrichment of *Irx3* binding and were able to identify direct binding targets of *Irx3*. These genes were filtered in Partek Genomics Suite. Using this program, we identified over 3000 genes using the Model-based Analysis of Tiling Arrays (MAT) score algorithm. GO enrichment identified relevant networks using an enrichment score and p-value. Referencing at Ensembl.org was then used to verify whether these genes conserved the *Irx3* consensus sequence across species. Using Interactive Genomics Viewer, we confirmed the presence of the repeated IRX3 consensus sequences. Finally, their respective gene pathways were mapped and confirmed using Ingenuity Pathway Analysis.

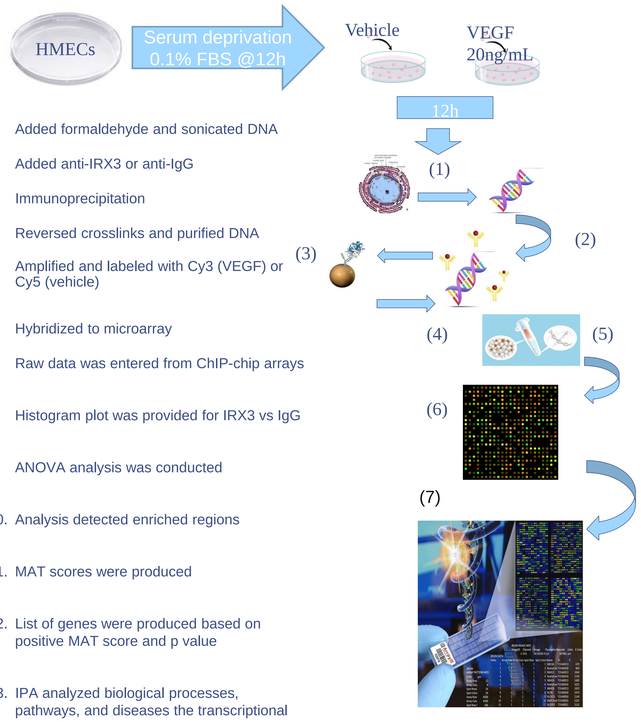
Introduction

Current research indicates a general knowledge of the process of angiogenesis and the transcription factor VEGF's involvement within it. This process includes signal transduction, involving VEGF's binding to tyrosine kinase receptors. As a specific mitogen for ECs, VEGF can stimulate EC proliferation, migration, and new blood vessel formation. The presence of a VEGF ligand gradient stimulates the specification of an EC leading cell into a "tip" cell, a cell with multiple filopodial extensions, or an EC "stalk" cell, a cell characterized with connectivity with the preexisting vessel. D114 and Notch Signaling control this process, determining which cells become "tip" or "stalk" cells. Previous literature indicates increased levels of D114 and VEGFR2 in tip cells as well as increased levels of Notch and VEGFR1 in stalk cells.

IRX3 plays a critical role in proper angiogenesis in response to the VEGF ligand. In humans, IRX3 genes reside in two clusters of three genes each that encode transcription factors that recognize the unique palindromic DNA binding motif 5' -ACAnnTGT-3'. *Irx3* promotes EC tip cell specification as a downstream target of Notch signaling. Through this, *Irx3* mediates Human Microvascular Endothelial Cell migration. Here, we have determined the downstream targets of *Irx3* during angiogenesis through ChIP-on-chip experiment and bioinformatics analyses.

Current literature does not illustrate the significance of *Irx3* and its binding targets in relation to an angiogenic function. These binding targets could heavily influence angiogenesis due to their relation with *Irx3* and, by extension, VEGF. The objective of this study is to identify known as well as unknown 'pro-' and 'anti'-angiogenic novel target genes that bind to *Irx3* during VEGF-induced angiogenesis. This research aims to illuminate co-factors of *Irx3* to analyze their roles in VEGF-induced angiogenesis in relation to *Irx3*.

Methods



1. Added formaldehyde and sonicated DNA
2. Added anti-IRX3 or anti-IgG
3. Immunoprecipitation
4. Reversed crosslinks and purified DNA
5. Amplified and labeled with Cy3 (VEGF) or Cy5 (vehicle)
6. Hybridized to microarray
7. Raw data was entered from ChIP-chip arrays
8. Histogram plot was provided for IRX3 vs IgG
9. ANOVA analysis was conducted
10. Analysis detected enriched regions
11. MAT scores were produced
12. List of genes were produced based on positive MAT score and p value
13. IPA analyzed biological processes, pathways, and diseases the transcriptional regulators and their targets may control, and how these upstream molecules may regulate one another

Results

Ingenuity Pathway Analysis

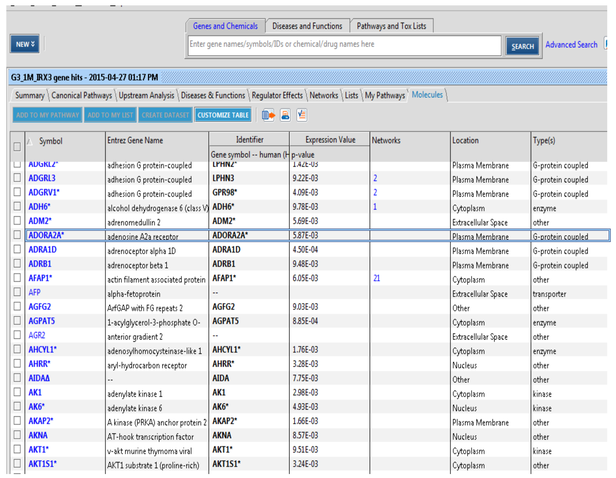


Figure 1: Ingenuity Pathway Analysis (IPA) identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes in our dataset. This program helps illuminate the biological activities and factors that regulate HMVECs on a genomic level.

Ingenuity Pathway Analysis Canonical Graph

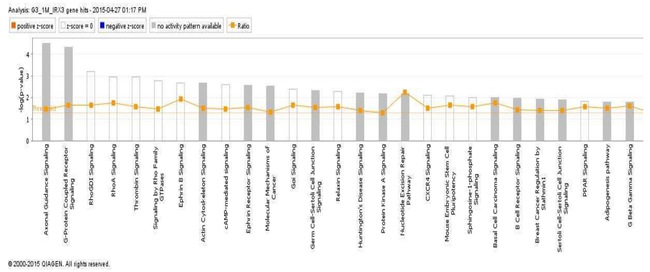


Figure 2: Ingenuity Pathway Analysis (IPA) used prior knowledge of expected effects between transcriptional regulators and their target genes stored in the Ingenuity Knowledge Base to identify the top canonical pathways.

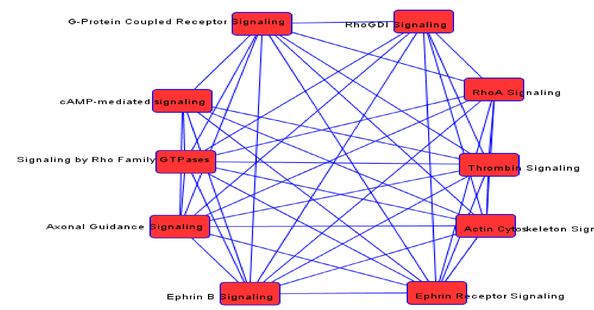
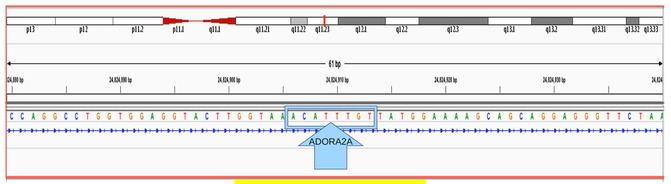


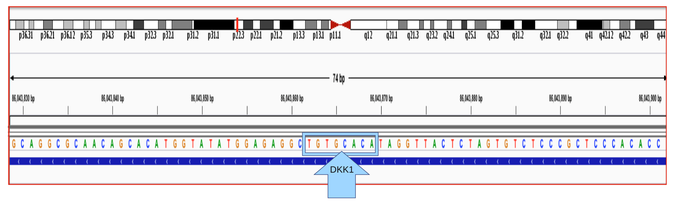
Figure 3: Ingenuity Pathway Analysis (IPA) displays canonical networks and emphasizes how each specific pathway is interrelated. IPA can also highlight the most significant genes associated with each of these unique pathways.

Integrative Genomic Viewer



Consensus Sequence

Figure 4: Integrative Genomic Viewer (igv) uses preexisting literature to locate the gene of interest to a specific chromosome while also identifying the consensus sequence for *Irx3* in *Adora2A.*



Consensus Sequence

Figure 5: Integrative Genomic Viewer (igv) uses preexisting databases to identify the specific genes of interest while simultaneously localizing them to their specific chromatin. The *Irx3* consensus sequence is also located in *Dkk1.*

Conservation Across Species

		Distance from TSS		5' Flank	Core Consensus	3' Flank
ADORA2A	Human	4551 nt 3181 nt 2967 nt		5'-TGTGAGCAGC 5'- TATGGAAAAG 5'- GAGGGCCTTG	TGTGTACA ACATTTGT TGTGTACA	CACCACAGCC-3' TACTTGCTAA-3' CATCTGGGCA-3'
	Mouse	4934 nt 2525 nt 495 nt	862 nt	5'-ATGCACGAAG 5'- GTACCACATC 5'- CAGGCACACC 5'- GATTGAGATT	TGTCTACA TGTCTACA TGTAGACA TGTGAACA	TTAGAGTGCC-3' GCTGTTTTTC-3' TAACCAGATC-3' CTTGCCCTGT-3'
	Rat	3219 nt 2439 nt		5'-GTCTCAGGCT 5'- AGCCCCTAGT	TGTGAACA TGTACACA	AGTGGTCAGA-3' GCTTTGGACT-3'

Figure 6: The IRX3 Consensus Sequence for ADORA2A is repeated and conserved across multiple species within 5 Kb of the Transcriptional Start Site.

Discussion

The pilot study for ChIP-on-chip was a 243K array that identified 871 genes directly associated with Irx3; the million feature array identified over 3,000 genes. These results were filtered with a positive MAT score and p-value of less than 0.05. This data was then cross-referenced with IPA and igv, which targeted 4 focus genes (ADORA2A, DKK1, EP300, and CYR61). Each of these genes contained the IRX3 consensus sequence ACAnnTGT. These consensus sequences were conserved across species (e.g., human, rat, and mouse) and were located within 5000 base pairs of the Transcriptional Start Site. As each gene contained repeated IRX3 consensus sequence, it is probable that preferential binding is occurring between IRX3 and each gene.

Adenosine Receptor A2a (ADORA2A) works with Toll-like receptor (TLR) to switch macrophages from production of inflammatory cytokines such as tumor necrosis factor-alpha to production of VEGF and Thrombospondin-1 (TSP-1) [1-6]. A knockdown of ADORA2A could prevent production of TSP-1 and VEGF, inhibiting angiogenesis. Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) is able to down-regulate the micro-vessel density and VEGF expression [1-4]. Through this, DKK1 is able to decrease the expression level of endothelial marker Pecam1 [6]. E1A Binding Protein P300 (EP300) is a co-regulator of HIF1A (hypoxia-inducible factor 1 alpha), and therefore is a key regulator of hypoxia-induced genes such as VEGF [8]. Its effect on VEGF relies on EP300 acetylation, which allows receptor phosphorylation and intracellular signaling upon prolonged stimulation with VEGF [10]. Cysteine-Rich 61 (CYR61) increases both cell migration and proliferation, inducing angiogenesis [3]. CYR61 has both direct and indirect effects on VEGF by through inducing ECs to promote all the steps of angiogenesis and up-regulating VEGF [9]. As each of these genes contained repeated IRX3 consensus sequence, it is likely that each gene co-regulates angiogenesis with IRX3. Mapping the genetic regulatory network allows researchers and clinicians to gain deeper understanding of angiogenesis.

Future treatments will rely upon genomic intervention to accelerate treatment as well as recovery time following the onset of pathologic conditions associated with angiogenesis. The identification of these genes can be implemented into therapeutic regimes for patients. The ability to regulate the stimulation and inhibition of angiogenesis could decrease both morbidity and mortality rates in certain diseases associated with this process. Following a myocardial infarction, the stimulation of angiogenesis could recreate new blood vessels to compensate for the damaged vessels, speeding recovery up enormously. Conversely, the prevention of tumor metastasis could be achieved by inhibiting angiogenesis around the tumor to prevent its growth and progression. Peripheral Vascular Disease (PVD) could be avoided by stimulating the growth of new blood vessels specific to areas suffering from ischemia. This genomic intervention could have implications on effectiveness and cost of treatments. In the event of a stroke within the transverse aorta, the stimulation of angiogenesis could create new blood vessels to circumvent the blocked valve and provide nutrients to the damaged area. Each treatment would also reduce the patient's stay within the hospital, lessening the cost of treatment. Therefore, this data could help develop future treatments, reducing the morbidity rates of cardiovascular diseases, increase the treatment's effectiveness, decrease hospital duration length, and prove to be cost effective. The next step to achieving that treatment is moving from bioinformatics identification of these co-regulators to *in vitro* and *in vivo* experiments to determine if and how overexpression or knockdown of these genes affects angiogenesis.

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