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NUTS AND BOLTS

Temporal VEGFA responsive genes in HUVECs: Gene signatures and potential ligands/receptors fine-tuning angiogenesis

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Abstract

Vascular Endothelial Growth Factor-A (VEGFA) signaling is crucial to the cellular processes involved in angiogenesis. Previously, we assembled a network of molecular reactions induced by VEGFA in human umbilical vein endothelial cell populations. Considering transcriptome as a read-out of the transcriptional and epigenomic regulatory network, we now present an analysis of VEGFA-induced temporal transcriptome datasets from 6 non-synchronized studies. From these datasets, applying a confidence criterion, a set of early VEGFA-responsive signature genes were derived and evaluated for their co-expression potential with respect to multiple cancer gene expression datasets. Further, inclusive of a set of ligand-receptor pairs, a list of ligand and receptor signaling systems that potentially fine-tune the endothelial cell functions subsequent to VEGFA signaling were also derived. We believe that a number of these signaling systems would concurrently and/or hierarchically fine-tune the signaling network of endothelial cell populations towards the processes associated with angiogenesis through autocrine, paracrine, juxtacrine, and matricrine modes. By further analysis of published literature on VEGFA signaling, we also present an improved update-version of our previous VEGFA signaling network model in endothelial cells as a platform for analysis of cross-talk with these signaling systems.

Keywords Angiogenesis · VEGF · Gene expression · HUVECs · Cross-talk · Signature

Abbreviations

DEGs	Differentially Expressed Genes
EC	Endothelial cell
EGF	Epidermal Growth Factor
ENA	European Nucleotide Archive
FC	Fold Change
FGF	Fibroblast Growth Factor
GEO	Gene Expression Omnibus

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GO	Gene Ontology
HUVECs	Human Umbilical Vein Endothelial Cells
MMP	Matrix MetalloProteinase
PTMs	Post Translational Modifications
TF	Transcription Factor
TGF-beta	Transforming Growth Factor-beta
VCAM	Vascular Cell Adhesion Molecule
VEGFA	Vascular Endothelial Growth Factor-A
VEGFR	Vascular Endothelial Growth Factor Receptor

Introduction

Angiogenesis, the process of formation of new blood vessels, is fundamental to numerous physiological processes such as development, wound healing, bone formation and female reproductive cycle (Caplan 1985; Reynolds et al. 1992; Gerber and Ferrara 2000; Tonnesen et al. 2000; Chung and Ferrara 2011; Portal-Núñez et al. 2012) as well as diverse pathological processes such as cancer, diabetic retinopathy, psoriasis and inflammatory diseases. (Folkman 1972a; Carmeliet and Jain 2000; Creamer et al. 2002; Nishida et al. 2006; Szekanecz and Koch 2007; Crawford et al. 2009). A fine balance of angiogenesis is maintained by pro-angiogenic factors and anti-angiogenic factors (Folkman and Klagsbrun 1987; Folkman and Shing 1992). Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF) Transforming Growth Factor (TGF), Epidermal Growth Factor (EGF) and thrombin are among the pro-angiogenic factors while Angiostatin, Endostatin and Thrombospondin-1 are among the anti-angiogenic factors (Otrock et al. 2007a; Karamysheva 2008). They could be further categorized into factors that mainly modulate EC proliferation and/or migration and factors that modulate structure and function of basement membrane and extracellular matrix (Bouïs et al. 2006).

VEGF, which exists in multiple isoforms is a critical modulator of endothelial cell (EC) proliferation and/or migration and is the best characterized growth factor involved in blood vessel development (Hoeben et al. 2004; Holmes and Zachary 2005; Roskoski 2007). They exert their biological function through interaction with their specific VEGF receptors, a family of transmembrane tyrosine kinase receptors. VEGF family comprises of VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and VEGFF with binding potential to the VEGF family of receptors, VEGFR1, VEGFR2 and VEGFR3 (Ferrara et al. 1992; Ferrara 1999). Among them, VEGFA-VEGFR2 forms the primary axis as mediators of angiogenesis. Beyond their role in proliferation, sprouting and tube formation, VEGFA also serves as a potent survival factor for ECs. VEGFA binding to VEGFR2 results in dimerization and autophosphorylation of their cytoplasmic domain (Otrock et al. 2007b) that subsequently initiates a network of signaling events in ECs relevant to angiogenesis (Reviewed and assembled by our group, Abhinand et al. 2016). Deregulation of angiogenesis leads to excessive, insufficient or abnormal processes associated with a number of disorders (Carmeliet 2003). Many treatment trials involving pro-angiogenic and anti-angiogenic treatments (Folkman 1972b) including anti-VEGF therapy were developed for diseases including cancer (Ferrara 2004; Al-Husein et al. 2012). A better understanding of the modulators of signal transduction events associated with angiogenesis is crucial for combinatorial therapy approaches to reduce side effects and drug resistance in patient cohorts (Carmeliet and Jain 2011; Yoo and Kwon 2013).

Human umbilical vein endothelial cells (HUVECs) had been extensively used to study molecular mechanisms underlying the EC proliferation and migration and also as an *in vitro* model for the development and analysis of anti-angiogenic therapies for cancer and other diseases (Rhim et al. 1998; Onat et al. 2011). The influence of proteins, their PTMs and interactions on processes such as proliferation and migration of HUVECs in response to VEGFA has served as a reference platform to study angiogenesis and its related diseases such as atherosclerosis, cerebral infarction, ischemia and cancers. (Favot et al. 2003; Beck and Plate 2009). Global temporal analysis of mRNAs is better scalable to RNA-sequencing platforms than that of the more diverse array of transient post-translationally modified forms of proteins by mass spectrometry. A number of gene profiling studies had been attempted to measure dynamic gene expression changes induced by VEGFA in EC models *in vitro* (Schoenfeld et al. 2004; Schweighofer et al. 2009). Despite the availability of these datasets, there had been no efforts to derive and analyze consistent VEGFA transcriptional targets. As temporal read-out through transcriptional and epigenomic regulatory network, an assembly and analysis of VEGFA-induced transcriptome of endothelial cells from multiple studies would also contribute to the identification of other ligand/receptor signaling systems with the potential to modulate the process of angiogenesis subsequent to VEGFA signaling. The present study introduces a set of EC signature genes of VEGFA signaling based on multiple temporal gene expression datasets longitudinal to time and consistency across them. Subsequently, based on EC transcriptional response to VEGFA, we also enlist a set of ligand-receptor systems that has potential to act through autocrine or paracrine modes and modulate endothelial-signaling network towards EC functions and angiogenesis. Further, we have also expanded the comprehensive map of VEGFA-VEGFR2 signaling previously published by our group (Abhinand et al. 2016) with data published subsequently in 2 years to enhance the analysis of the VEGFA-VEGFR2 signaling network and their potential cross-talk with other signaling systems.

Materials and methods

Datasets for VEGFA induced mRNA analysis

Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was searched for publically available datasets, with the keywords, "HUVEC", "VEGF treated", "Expression profiling by array" OR "Expression profiling by high throughput sequencing" [DataSet type] AND "gse" [Filter]. From the 18 hits obtained, datasets with expression profiles of VEGFA treated HUVECs as compared to untreated HUVECs were only considered. Accordingly, six datasets (microarray datasets- GSE837, GSE10778, GSE15464, GSE49426 and GSE53550, and RNA-Seq data set-GSE71216) were chosen for the current analysis.

Data preprocessing and DEG analysis

Raw data for GSE10778, GSE15464 and GSE49426 (CEL files) were downloaded from GEO database and was subjected to background correction and normalization using the GeneChip Robust Multiarray Average (GC-RMA) algorithm package in R language. Further pre-processing was done using the simpleaffy package for call detection and removal of control probes. Each of the probes in the dataset was mapped to its corresponding gene based on the platform information provided in the GEO database. When several probes were mapped to a single gene, the average of the expression values were calculated and considered it as the expression value of that gene. GSE837 and GSE53550 datasets were processed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) for the identification of DEGs as CEL files were not available for them. A p value ≤ 0.05 and $|\log_2 FC| \geq 1$ were set as the cut-off criteria for defining the differentially expressed genes (DEGs). For GSE53550, the probe annotations were not provided along with the platform in GEO and they were obtained from Gemma (https://gemma.msl.ubc.ca/home.html). Entrez Gene ID and Gene Symbol corresponding to the probes were mapped and used for further analysis.

FASTQ file of the paired-end raw reads of the RNA-Seq data [GSE71216] was downloaded from ENA and the quality assessment for the raw reads was carried out with FastQC(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The low-quality reads and adapter contamination were removed by using Trimmomatic-0.36 (Bolger et al. 2014). The trimmed reads were again subjected to quality assessment using FastQC and then mapped to the human reference genome hg38 build of UCSC, downloaded from Illumina iGenome website (https://support.illumina.com/sequencing/sequencing_software/igenome.html) by utilizing the TopHat2 tool (version 2.1.1) (Kim et al. 2013). The mapped reads were then subjected to differential expression analysis for the identification of DEGs between the VEGFA-treated and untreated HUVECs, using the Cuffdiff software (version 2.2.1) (Trapnell et al. 2013). Genes that satisfy a p value cutoff ≤ 0.05 and $|\log_2 FC| \geq 1$ were selected as significant differentially expressed genes.

Functional categorization of VEGFA-induced temporal DEGs

The DEGs from the VEGFA-induced transcriptome datasets were categorized into three time frames namely, early (0–1 h), intermediate (1–6 h) and late genes (after 12 h) based on the availability of the temporal data for gene set enrichment analysis. As the time point of 4 days in the RNA-Seq dataset GSE71216 was an outlier compared with all the other datasets, it was excluded from further analysis of temporal DEGs. Gene Ontology (GO) enrichment analysis of the DEGs were carried out using the DAVID tool (version 6.8) (Huang et al. 2007, 2009). A count number ≥ 5 genes and a p value ≤ 0.01 were used as the cut-off criterion for statistically significant GO terms associated with the DEGs.

Categorization of VEGF signature genes and their correlation with cancer gene expression datasets

A set of genes that were consistently responsive to VEGFA signaling at early time frames in the available datasets is characterized as "VEGFA signature genes". More specifically, the genes that are consistently up-regulated or down-regulated in two or more early time frame datasets or in both early and intermediate time frame in a dataset were categorized as VEGFA signature genes. Up-regulated signature genes were analyzed for correlation with the cancer gene expression datasets (breast cancer and lung cancer) available in cBioPortal (Cerami et al. 2012). Positively correlated top 100 co-expressed genes of each of the up-regulated signature genes were obtained.

Reconstruction of the VEGFA signaling pathway network

We improved our previously constructed map by following the annotation criteria discussed earlier (Abhinand et al. 2016. The newly added VEGFA- induced protein-protein interactions, enzyme-substrate reactions and the post-translational modifications in ECs (Date of update: 12/20/2018) are represented using PathVisio (Kutmon et al. 2015). VEGFA- induced DEGs in ECs are also represented in this network map if either the transcriptions factor(s) or the signaling modules involved in their regulation are experimentally reported in research articles.

Results

Functional analysis of VEGFA-induced endothelial cell transcriptome datasets

From 6 distinct non-synchronous temporal studies, 13 temporal datasets available were chosen for analysis of the transcriptome induced by VEGFA in endothelial cells. Together, 2552 unique protein coding genes were differentially expressed by 2-fold (p value ≤0.05) at mRNA level (referred as DEGs) from all the datasets. These DEGs constituted 637 enzymes, 245 transcription factors, 28 cytokines, 345 DNA binding proteins, 28 growth factors, 367 signaling molecules, and 32 structural proteins (Fig. 1). Out of the 637 enzymes, 65 were kinases. Among these DEGs, 972 genes showed increased expression (up-regulated) and 1466 genes showed decreased expression (down-regulated) relative to respective unstimulated state. There was only 114 genes that were common in both the up-regulated and down-regulated DEGs among all the individual temporal datasets. The information on the datasets (Supplementary Table 1A**)** and the overlap of genes across multiple temporal datasets **(**Supplementary fig. 1**)** are provided.

Enrichment based on Gene Ontology (GO) distributed the biological processes associated with DEGs into inflammatory response, DNA-templated transcription, signal transduction, apoptotic process, protein phosphorylation, cell adhesion, cell proliferation, cell division, mitotic nuclear division, response to drug, and angiogenesis. Significantly attributable to angiogenesis processes, 106 DEGs were involved in apoptosis, 92 in cell adhesion, 80 in cell proliferation, 69 in inflammatory process, 40 in cell migration and 39 in extracellular matrix organization (Fig. 2). The signaling modules or pathways such as PI3K-Akt, FoxO, ErbB, Ras, MAPK, Rap1, TNF, NF-kappa B, p53, HIF-1, focal adhesion, chemokine, proteoglycans in cancer, purine metabolism, pyrimidine metabolism, and the hematopoietic cell lineage were enriched for unique DEGs. A set of 114 DEGs also mapped to the genes that were annotated by GO Consortium to "angiogenesis" and their associated terms (Supplementary Table 1B). Further, 31 unique DEGs from all datasets also overlapped with 84 genes of the Human Angiogenesis RT^2 Profiler™ PCR Array profiles by Qiagen (Supplementary Table 1C).

Characterization of temporal VEGFA-induced transcriptome of endothelial cells

Taking into account the different time points of VEGFA stimulation and heterogeneity across multiple studies, we next sought to characterize the temporal qualitative and quantitative features of DEGs. Towards this, we categorized the expression data into three time frames - early (up to 1 h), intermediate (1–6 h) and late (6–24 h) based on the duration of VEGFA stimulation of HUVECs (Supplementary Table 2). Across the datasets from 6 studies, we classified 494 unique DEGs into early, 1298 into intermediate and 1007 into the late time frame regulated genes. Among the 494 early DEGs, 246 were up-regulated and 243 were down-regulated, while 5 DEGs overlapped across the up-regulated and down-regulated gene sets from multiple datasets. In the intermediate time frame, 336 genes were up-regulated and 939 genes were down-regulated, while 23 genes overlapped among them. Out of the 1007 late genes, 539 genes were up-

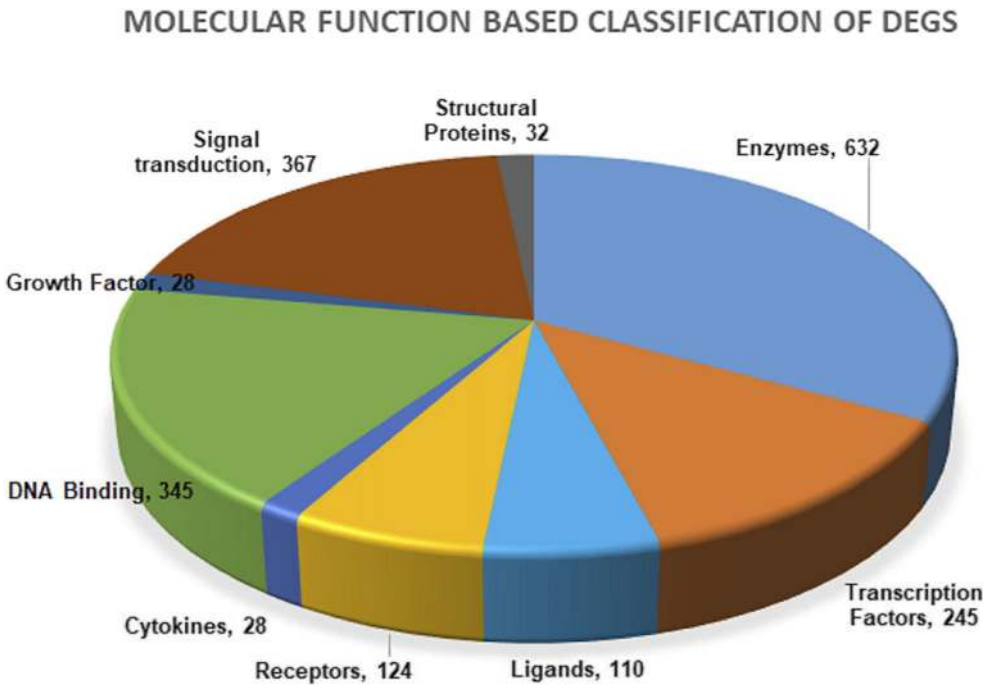

Fig. 1 Classification of VEGFA-induced DEGs. The major molecular functions enriched for the unique DEGs across all the temporal datasets from 6 studies are represented.

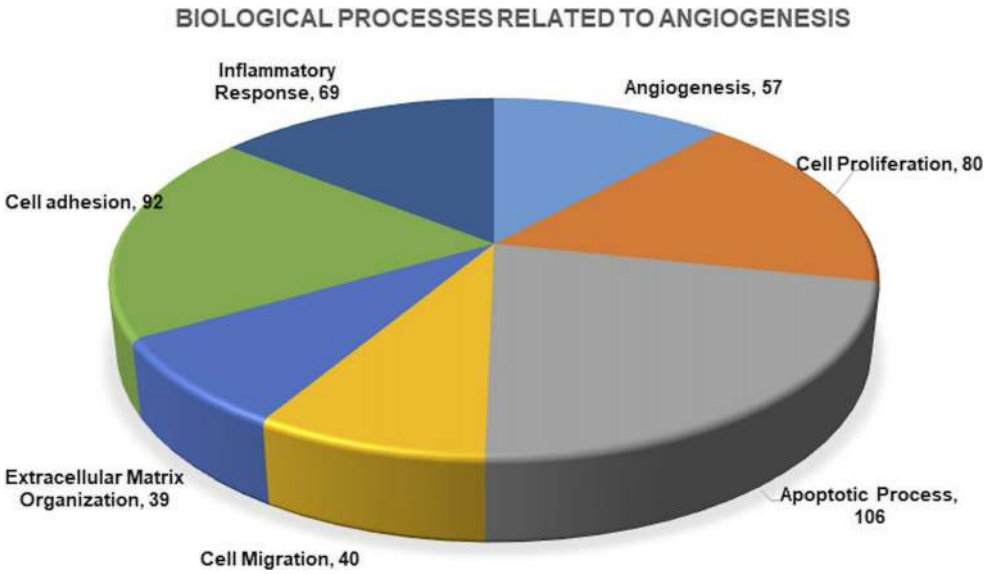

Fig. 2 Categorization of DEGs into GO-enriched biological processes associated with angiogenesis. The number of DEGs enriched for well known biological processes associated with angiogenesis such as extracellular matrix organization, inflammatory process, cell proliferation, cell adhesion, cell migration and apoptosis are represented.

regulated, 458 genes were down-regulated and 10 genes overlapped among them. Considerably, less than 3% of the DEGs were common across at least 2 up-regulated and down-regulated sets within the temporal frames.

Significantly, the biological processes associated to 494 early genes were skewed towards the 'inflammatory response', 'regulation of transcription' and other biological processes associated with angiogenesis GO terms (such as 'angiogenesis', 'vasculogenesis', 'cellular response to vascular endothelial growth factor stimulus', 'endothelial cell chemotaxis', 'positive regulation of endothelial cell proliferation', 'patterning of blood vessels', 'response to ischemia', and 'cell-adhesion'). Notably, the genes involved in ubiquitin-dependent protein catabolic process, especially protein deubiquitination, such as *USP5, USP8, USP25, USP34, USP45, USP44,* and *JOSD2* were mostly down-regulated within 1 h (Supplementary Table 3). At the intermediate and late time frames, these processes were scattered with other

Table 1 List of up-regulated and down-regulated VEGFA signature genes

97 Up-regulated Signature Genes				83 Down-regulated Signature Genes			
AASS	DUSP1	JUNB	PER2	AKAP10	GPR126	PRPF38B	ZNF160
ACKR3	DUSP10	KCDT3	PPAP2B	APEX2	GTF2H2	PTPN13	ZNF207
ADAMTS1	DUSP5	KCNJ2	PRKD2	ASCC2	GTF2H3	RAB11FIP3	ZNF431
AFAP1	EGR1	KITLG	PROX1	ATP8B1	HCFC2	RSRP1	ZNF611
ARHGAP24	EGR3	KLF10	PSG1	BBS9	HNRNPA2B1	RUNX1T1	ZNF638
ARL17A	ELF1	KLF2	PTGS2	C11orf54	INTS2	RYR3	ZNF721
ATF1	ERBB4	KLF4	RAPGEF5	C11orf80	KAZN	SAMD12	ZNF862
ATF3	FBXO5	LBH	RCAN1	C9orf64	KRT8	SAMD9	ZXDB
BHLHE40	FCRL4	MAFF	RGS2	CCDC152	LCORL	SDHA	
BMP2	FJX1	MAFK	SCD5	CDK13	LPPR4	SLC25A37	
C8orf4	FLG	MAP3K8	SELE	CDK5RAP3	LRIG2	SLC35D2	
CCL2	FOSL2	MCL1	SGK1	CSNK1A1	LYRM5	SLC44A5	
CCPG1	FOXC1	MLPH	SOCS6	CYBRD1	MITD1	SUDS3	
CEBPD	GEM	MRPL44	SPRY2	DIP2A	MTUS1	TAF15	
CITED2	GPSM2	NCKAP1	TNFAIP3	DLGAP4	NDUFAF3	TAF1D	
CLCA2	HBEGF	NDRG1	TNFAIP8L3	DOCK4	NDUFS8	TATDN3	
CNKSR3	HERPUD1	NEDD9	TRIB1	E2F8	NOTCH2NL	TRA2A	
COLCA1	HEY1	NFIL3	TRIM4	EDN1	NPIPA1	TXNIP	
CREB5	HIVEP2	NFKBIA	VCAM1	EIF3B	NPIPA5	USP25	
CREM	ICAM1	NFKBIZ	VEGFA	FBXW12	NUMBL	USP34	
CSRNP1	IER2	NOV	WDR87	FIGN	PCNX	USP44	
CXCL1	IGFBP3	NR4A2	ZNF492	FRMD4A	PECR	USP5	
CXCL2	IL6	OPHN1		GALNT6	POGZ	VPS13C	
CXCL8	IPMK	PAQR3		GJA5	PPDPF	XPO1	
DNAJB9	ITPKB	PCDH17		GPM6A	PPP1R16B	ZBTB26	

biological processes. Among the 494 early genes, 112 were enzymes, 77 transcription factors, 23 ligands, 13 receptors, 8 cytokines, 101 DNA binding proteins, 8 growth factors, 58 signal transduction molecules and 2 structural proteins. This has prompted further analysis of specificity of VEGFA signaling regulated genes in the early time frame.

VEGFA signature gene set and their co-expression analysis in cancers

We analyzed the pattern of expression of DEGs in response to VEGFA across the multi-study datasets. There were no DEGs consistent and common across all these datasets. However, a set of 119 and 141 DEGs were consistently up-regulated and down-regulated, respectively, in 2 or more temporal datasets across one or more of the 3 temporal frames (Supplementary Fig. 2). Among these genes, 10 or more up-regulated and down-regulated genes are encoded in chromosomes 1, 7, and 19. Considering multiple temporal datasets from individual study as synchronous and each temporal datasets across multiple studies as non-synchronous to each other, we applied a selection criterion to obtain a set of high-confidence early VEGFA responsive genes. Genes that were either up-regulated or down-regulated consistently in two or more datasets in the early time frame, or in both the early and intermediate time frame in temporal datasets within an individual study, were categorized as early VEGFA signature genes. Following this criterion, we identified 97 up-regulated and 83 down-regulated genes as early VEGFA signature genes (Table 1). Although, time frame up to one-hour time is currently considered as early, this could be subsequently dissected based on further data availability in the future. For a number of these genes such as *DUSP1/5*, *EGR3*, *NR4A2, HERPUD1* and *DNAJB9* their responsiveness to VEGFA within the early time frame were also experimentally validated using qPCR in endothelial cells (Bellou et al. 2009; Schweighofer et al. 2009; Karali et al. 2014; Mena et al. 2014). Analysis of the transcription factors (TFs) associated to individual up-regulated signature genes obtained from ChipBase (Zhou et al. 2016) identified 16 TFs that were annotated in our assembled VEGFA signaling pathway model (Abhinand et al. 2016).

In order to ascertain the co-expression potential and significance of VEGFA signature genes, the cancer gene expression datasets available in cBioPortal (Cerami et al. 2012) were analyzed for the positively correlated genes of the up-regulated signature genes (considering angiogenesis as a hallmark of cancer). Top 100 positively correlated genes of each of the up-regulated VEGFA signature genes were filtered from the breast and lung cancer datasets. There were 17 and 18 individual VEGFA signature genes with more than 10 co-expressed genes within the VEGFA signature set in breast and lung cancer gene expression datasets, respectively. A set of 11 genes among them, namely *EGR1, IL6, CXCL2, TNFAIP3, DUSP1, ATF3, RGS2, ADAMTS1, HBEGF, JUNB* and *CSRNP1*, were common between the two cancer datasets. This attributes the co-expression potential of the early VEGFA signature genes derived from multiple datasets and also their relevance to angiogenesis in multiple cancers.

Potential intrinsic and extrinsic fine-tuners of VEGFA signaling network

The ligand/receptor signaling systems that fine-tune endothelial cell functions associated to angiogenesis subsequent to VEGFA signaling are poorly defined. As an initial step towards this, we attempted to delineate ligand and receptor mRNAs regulated by VEGFA at multiple time frames. The known/characterized ligands, receptors and the ligand-receptor pairs in the VEGFA-responsive DEGs from temporal datasets were filtered based on the assembled data by Ramilowski et al. 2015 (Ramilowski et al. 2015). We delineated 110 ligand/stimuli and 124 cell surface receptor mRNAs that were differentially regulated by VEGFA (Supplementary Table 4) in the analyzed datasets. We also identified 48 known ligand-receptor pair combinations that could be visualized towards their potential autocrine mode of signaling. Among them, CCL2-ACKR4, CXCL2-CXCR2, HBEGF-ERBB4, PPBP-CXCR2 and VEGFA-FLT1 pairs were up-regulated within the 6 h time frame of VEGFA stimulation suggesting their prominent involvement in the subsequent fine-tuning of the EC signaling network in an autocrine manner (Supplementary Table 4A).

KEGG pathway enrichment of the ligands and receptors visualized cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, Rap1 signaling pathway, ECM-receptor interaction, Chemokine signaling pathway, Focal adhesion, TNF signaling pathway, Ras signaling pathway, Pathways in cancer, ErbB signaling pathway, MicroRNAs in cancer, Proteoglycans in cancer, HIF-1 signaling pathway, Notch signaling pathway and NOD-like receptor signaling pathway. Notably, along with other non-ligand and non-receptor genes, these pathways were already enriched by the unique VEGFA responsive DEGs.

Cross-talk potential of ligand-receptor systems on kinases

XTalkDB database (Sam et al. 2016) enlists TGF-beta, wnt, hedgehog, estrogen, thyroid hormone, neurotropin and GnRH signaling pathways to cross-talk with VEGFA signaling pathway. With 110 ligands and 124 receptors of potential autocrine or paracrine mode of signaling, we next focused on the ligand-receptor signaling systems transcriptionally regulated by VEGFA for their potential to

modulate kinases that are already associated to the VEGFA signaling pathway and also those transcriptionally regulated by VEGFA signaling. The kinases in the DEGs were filtered from the KinBase (Manning et al. 2002). Surprisingly, out of the 65 kinases identified in VEGFA-induced DEGs, only 11 kinases (namely BRD4, BMX, CSK, FLT1, FYN, MAP2K6, MAP2K7, PAK1, PRKD1, PRKD2 and ABL1), were associated to our assembled VEGFA signaling pathway. We enlisted as many as 54 kinases from the VEGFA-induced DEGs that have not been previously associated with VEGFA signaling pathway. This demands their further investigation either as mediators of VEGFA signaling or as critical players of other signaling systems that subsequently fine-tune endothelial cell functions relevant to angiogenesis. Towards the analysis of other ligand-receptor systems to potentially modulate these kinases, a protein-protein interaction network of 65 kinases, 31 ligands (for which at least one of their known receptors were identified in VEGFA-induced DEGs) and 124 receptors was developed based on the STRING database (Szklarczyk et al. 2014). Among receptors and kinases in the DEGs of VEGFA, 24 kinases were direct interaction partners of 19 receptors together forming around 75 interactions (Supplementary Fig. 3). This attributes the potential role of these ligand/receptor systems and kinases differentially regulated by VEGFA to subsequently fine-tune the EC signaling network.

Reconstructed network of VEGFA signaling pathway

We have also enhanced the pathway map that was previously published (Abhinand et al. 2016). From over 2000 research articles pertaining to VEGF signaling published since 2016, our manual curation approach resulted in the identification of 174 protein-protein interactions, 3 post-translational modifications, 13 activation-inhibition reactions and 29 DEGs induced by VEGFA in ECs. The pathway data in BioPAX and GPML formats is made available through WikiPathways (https://www.wikipathways.org/index.php/Pathway:WP3888) for visualization, enrichment analysis, customization and representation. Together, this improved pathway map represents 438 unique proteins compared to the 240 proteins in the previous version (Fig. 3). Complete set of VEGFA signaling network along with the currently appended reactions are provided in supplementary Table 5.

Discussion

The VEGFA-VEGFR2 axis is one of the critical signaling systems involved in the process of angiogenesis. It is a coordinated process regulated by multiple extrinsic signaling systems of endothelial cells. Hence, it is evident that a temporal regulation of the signaling events in ECs that drive angiogenesis are synchronized to a series of extrinsic signaling systems and their regulated gene expression profiles. Though gene expression is conceived stochastic to cell populations, the stochasticity is also attributed to phenotypic heterogeneity of cell populations in the tissue microenvironment (Huang 2009; Yuan et al. 2016). Hence, the DEGs identified from cell culture systems in response to stimuli may be considered as average fold change relative to a compared state, mostly the unstimulated state. Indicative of an active transcriptional reprogramming initiated by VEGFA, 77 DEGs induced within 1 h in ECs were part of the transcription regulatory network (such as ERG3, ELF1, FOSL2, ATF1/3 and FOXC1) and the histone acetyl transferases such as *KAT8*, *OGT* and *PHF20* were part of the epigenetic regulatory network . Implying the relevance of our temporal categorization of multiple gene expression datasets towards the analysis potential of VEGFA-induced pathway, less than 3% of the DEGs spanned both up-regulated and down-regulated categories in 2 or more datasets within specific temporal frames. Applying a confidence criterion to early temporal datasets longitudinal to time and consistency of regulation across datasets, we have also categorized a set of early signature genes that could be used as a reference for analysis of VEGFA responsiveness in endothelial cells. Many of these genes also showed a co-regulation potential within the datasets and also across an array of breast and lung cancer gene expression datasets.

A major challenge associated with the temporal dynamic analysis of signaling events and gene expression in response to any stimuli is the influence of cross-talk and fine-tuning by concurrent/hierarchical signaling systems. Towards an initiative to identify such potential signaling systems that could fine-tune endothelial signaling network subsequent to VEGFA signaling, based on the temporal VEGFA-induced gene expression profiles, we have derived a list of 110 ligands and 124 receptors including 48 ligand-receptor pairs of potential autocrine mode of action. As downstream signaling systems induced by VEGFA stimulation in ECs, the focus till now has been mainly on a limited number of signaling systems such as Notch (Liu et al. 2003), CXCL8 (Charalambous et al. 2005), Integrin beta-3/beta-5/alpha-V (Suzuma et al. 1998), and Sphingosine 1-phosphate receptor (Igarashi et al. 2003). For a large share of potential ligand/receptor systems derived from the current analysis, their role in endothelial cell functions is poorly defined. At the intracellular signaling network level, surprisingly, only 11 out of the 65 kinases transcriptionally regulated by VEGFA across multiple time point datasets were observed in our comprehensive VEGFA signaling pathway model. The significance and the role of other 54 kinases including VRKs, ATM, AAK1, BUB1 and NEK2 in VEGFA signaling network or subsequent hierarchical signaling by downstream signaling systems of VEGFA demands further investigation.

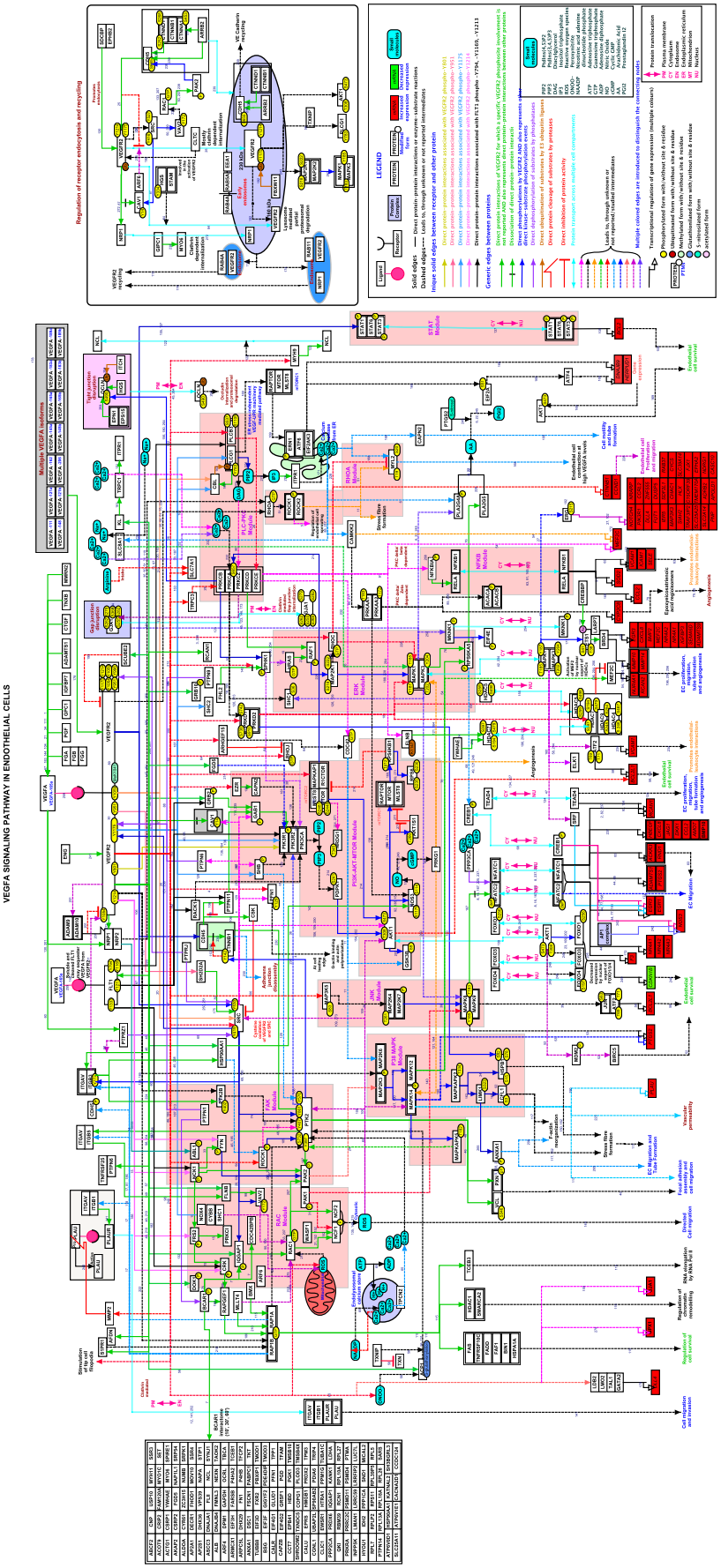

Fig. 3 An improved and update version of the VEGFA/VEGFR2 signaling network model. The proteins and their experimentally identified signaling network reactions characterized into protein-protein interactions, catalytic reactions, activation/inhibition reactions and translocation events induced in response to VEGFA in endothelial cells are represented in accordance with our previous model (Abhinand et al. 2016). VEGFA-induced DEGs in ECs for which experimental evidence was available on either the transcription factor(s) or the signaling modules involved in their regulation are also represented. The information on the nodes, edges, and color codes are provided for reference and efficient navigation of the pathway model. The information on the proteins or DEGs and the research articles with experimental evidence for each of the reactions can be obtained from the gpml file visualizable through the Pathvisio tool and can be used for customization and representation of experimental data pertaining to VEGFA signaling pathway by the scientific community.

DEGs obtained from each of the datasets were also compared with 24 h VEGFA-induced differential proteome of HUVECs analyzed by Mohr et al. 2017 (Mohr et al. 2017), in extracellular (up-regulated: 61, down-regulated: 40), cytoplasmic (up-regulated: 206, down-regulated: 133) and nuclear fractions (up-regulated: 66, down-regulated: 70) by LC-MS/MS analysis. Observing the lack of correlation of expression between VEGFA induced mRNAs and proteins in the early and intermediate time frames, Mohr et al. 2017 had proposed a pre-programing by VEGFA first towards inflammatory response and then further by necessary co-stimulations. Towards this, our analysis of the stimulatory layers (cross-talk at ligand/receptor level with the initial VEGFA signaling network) is intriguing and seeks further investigation.

VEGF ligand-focused therapeutic approaches have not succeeded to a desirable extend and hence, an analysis of cross-talk and complementary influence of other ligands towards angiogenesis is warranted to improve the efficacy of VEGF therapy. The list of ligands/receptors obtained through our approach, in future, could pave way into focused analysis of large spectrum of soluble factors or juxtacrine and matricrine factors in the endothelial cell microenvironment with the potential to modulate angiogenesis. Intriguingly, the analysis of concurrent or hierarchical expression of ligands or receptors in response to VEGFA would lead to better characterization of their functional roles to define a temporal regulatory map of angiogenesis events in the future. Towards this perspective, VEGFA also regulated 19 genes (up-regulated: 16 and down-regulated: 3) including interleukin-6 (IL-6), toll-like receptor 3 (TLR3) and interleukin-1 receptor associated kinase 2 (IRAK2) that are associated with inflammatory response, potentially to involve leukocyte recruitment as an essential early event. Hence, this study also opens up the analysis of transcriptional and epigenomic regulation of concurrent/hierarchical expression of ligand/receptor signaling systems. We believe that our approach to delineate ligand-receptor system-based early and/or consistent transcriptional targets as signatures and multi-dataset-integrated genes would serve as a reference platform for comparative analysis of individual signaling systems and cross-talk among them at temporal and cell type specific manner.

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