AOP ID and Title:

AOP 43: Disruption of VEGFR Signaling Leading to Developmental Defects
Short Title: Developmental Vascular Toxicity

Graphical Representation

MIE	KE1	KE2	KE3	AO
VegfR2 inhibition	Reduction in angiogenesis	Impairment of endothelial network	Vascular insufficiency	Developmental defects

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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	EAGMST Under Review	1.6	Included in OECD Work Plan

Abstract

BACKGROUND: The cardiovascular system is the first functional organ system to develop in the vertebrate embryo, reflecting its critical role during normal development and pregnancy. Elucidating an AOP for embryonic vascular disruption must consider the stepwise events underlying blood vessel patterning. Vascular development commences in the early embryo with in situ formation of nascent vessels from angioblasts, leading to a primary capillary plexus (vasculogenesis). After the onset of blood circulation, the primary vascular pattern is further expanded as new vessels sprout from pre-existing vessels (angiogenesis). Both processes, vasculogenesis and angiogenesis, are regulated by genetic signals and environmental factors dependent on anatomical region, physiological state, and developmental stage of the embryo. The developing vascular network is further shaped into a hierarchical system of arteries and veins, through progressive effects on blood vessel arborization, branching, and pruning (angioadaptation). These latter influences include hemodynamic forces, regional changes in blood flow, local metabolic demands and growth factor signals. Disruptions in embryonic vascular patterning-adaptation may result in adverse pregnancy outcomes, including birth defects, angiodysplasias and cardiovascular disease, intrauterine growth restriction or prenatal death. Some chemicals may act as potential vascular disrupting compounds (pVDCs) altering the expression, activity or function of molecular signals regulating blood vessel development and remodeling. Critical pathways involve receptor tyrosine kinases (e.g., growth factor-signaling), G-protein coupled receptors (e.g., chemokine signaling), and GPI-anchored receptors (e.g. uPAR system).

DESCRIPTION: This AOP focuses on the regulation and disruption of vasculogenesis-angiogenesis during embryonic development via disruption of the VEGF-signaling pathway. VEGFA binding to its cognate receptor (VEGFR2) triggers angiogenic sprouting, growth and fusion during early development, and in flow-sensing adaptation of vascular development during later development. VEGFR2 inhibition, the postulated molecular initiating event (MIE) for this AOP, may be invoked by effects on VEGFA production, mobility, or receptor binding, and by effects on VEGFR2 cellular expression, molecular function or post-receptor signal transduction pathways. Downstream key events (KE) include altered cell fate and behavior of 'endothelial tip cells' (exploratory behavior, cell migration) and endothelial 'stalk cells' (cell proliferation, apoptosis). KE relationships (KERs) leading to vascular insufficiency then involve local interactions with other cell types (stromal cells, macrophages), the extracellular matrix (ECM) and micro-physiology

(hemodynamics, metabolism). Adverse outcomes (AO) would ultimately vary by anatomical region, organ system, gestational stage and state of the embryo, fetus or placenta when an MIE is invoked.

RELEVANCE and APPLICATION: Angiogenesis and vascular disruption is a broad concept. The intended use of this AOP in a regulatory context is the predictive toxicology of developmental hazards, especially for integrating data from high-throughput screening (HTS) assays into cell agent-based models for predicting dysmorphogenesis. As part of an integrated assessment of toxicity, this AOP can identify useful information for assessing adverse outcomes relevant to risk assessment and efficient use of resources for validation through predictive models linking developmental toxicity to vascular disruption. AOP-based computer models that simulate vascular development can usher-in new virtual screening techniques to predict what might happen to a developing embryo when exposed to chemicals across different dose-time-stage scenarios, including the range of effects and how cellular injury propagates across development.

Background

https://aopwiki.org/wiki/index.php/File:KleinstreuerKnudsenAOPVascularDisruption.jpg

Functionalization of the ToxCast pVDC predictive signature

The ToxCast putative Vascular Disrupting Chemicals (pVDC) signature will be described here and parts will be incorporated into the relevant Key Events sections.

The sectors of the ToxPI are color-represented by features from ToxCast HTS assays indicated by the target of the assays, the characteristics as follows.

Vascular cell adhesion molecule 1 (VCAM1): the pVDC signature aggregates assays from the BioMAP Systems Predictive Toxicology panel [Houck et al., 2009, Kunkel et al., 2004] focusing here on chemical disruption of endothelial VCAM1 expression following stimulation by cytokines-growth factors. This assay endpoint is an in vitro surrogate for inflammatory cell recruitment per endothelial dysfunction and has been probed across five different cell systems: 4H (HUVECs stimulated with IL-4 + histamine); 3C (HUVECs stimulated with IL-1β + TNFα + IFNϒ); CASM3C (primary human coronary artery smooth muscle cells stimulated with IL-1β + TNFα + IFNϒ); LPS (HUVECs co-cultured with monocytes and stimulated with bacterial endotoxin); and hDFCGF (human dermal fibroblasts stimulated with IL-1β + TNFα + IFNϒ and EGF + bFGF + PDGF-BB)[Knudsen and Kleinstreuer, 2011, Kleinstreuer et al., 2014].

Angiogenic cytokines and chemokines: the pVDC signature aggregates features for LPS-induced TNFα protein expression (see BioMAP descriptor above), nuclear factor-kappa B (NFkB) mediated reporter gene activation (Attagene; cis- configuration), and caspase 8 enzymatic activity (NovaScreen; inhibition or activation). TNFα is a proinflammatory cytokine that can promote angiogenesis indirectly through NFkB-mediated expression of angiogenic growth factors, or inhibit angiogenesis by direct effects on endothelial proliferation and survival. The pVDC signature also aggregates features for signaling activity of the pro-angiogenic cytokines interleukin-1 alpha (IL1a, a macrophage-derived activator of TNFα) and interleukin 6 (IL6). These cytokines act through the G-protein coupled receptors (GPCRs) IL1R and IL6R, respectively. CXCL8 (chemokine (C-X-C motif) ligand 8), formerly known as interleukin 8 (IL8), is angiogenic through its cognate GPCRs (CXCR1, CXCR2). In contrast to CXCL8, the chemokines CXCL9 (alias MIG, monokine induced by IFNϒ) and CXCL10 (alias IP10, interferon-inducible cytokine IP-10) are considered anti-angiogenic through their cognate receptor, CXCR3.

Angiogenic growth factors: FGFs and VEGFs exert their effects on endothelial cell proliferation, migration, and differentiation via specific binding to receptor tyrosine kinases VEGFR and FGFR. The pVDC signature has features for liganding VEGFR1, VEGFR2, and VEGFR3 based on receptor kinase activity (RTK, inhibition or activation) from the NovaScreen biochemical profile [Sipes et al. 2013] and for down-regulation of VEGFR2 expression in the 4H BioMAP system (HUVECs stimulated with IL-4 + histamine, B). VEGFR1 is a non-signaling VEGF-A decoy receptor that can be cleaved from the cell surface; VEGFR2 is the most important VEGF-A receptor and a master switch for developmental angiogenesis; and VEGFR3 is a VEGF-C receptor up-regulated by Notch signals. The pVDC signature includes features for the basic helix-loop-helix transcription factors Aryl Hydrocarbon Receptor (AhR) and Hypoxia Inducible Factor-1 alpha (HIF1a) that are upstream regulators of VEGF gene expression during ischemia or hypoxia. HIF1a and AhR are measured in reporter assays (Attagene). In addition to HIF1a and AhR, the pVDC signature has features for the estrogen receptor alpha (ERa), also a trans-activator of VEGF expression. This included human ERa binding activity (NovaScreen), ERa reporter trans-activation (Attagene) and ERE (estrogen responsive element) reporter cis-activation (Attagene).

Angiogenic sprouting: the ephrins (EFNA1 and EFNB2 in particular) couple VEGF signaling to angiogenic sprouting during early development of the embryonic vasculature (vasculogenesis, angiogenesis). The ToxCast pVDC signature included features for EPH-receptor tyrosine kinase biochemical activity (increased or decreased) for receptors EPHA1, EPHA2, EPHB1 and EPHB2 via their cognate cell membrane-anchored ligands (EFNAs). In contrast to the ephrin system, a number of chemicals had activity on diverse assays for urokinase-type plasminogen activator (uPA). That system, consisting of uPA (4 features) and its GPI-anchored receptor, uPAR (8 features) - both assayed in the BioMAP System [Kleinstreuer et al. 2014], functions in VEGFR2-induced changes to focal adhesion and extracellular matrix (ECM) degradation at the leading edge of endothelial cells during angiogenic sprouting. Binding of uPA to uPAR results in serine-protease conversion of plasminogen to plasmin that initiates a proteolytic cascade leading to degradation of the basement membrane and angiogenic sprouting. The uPA proteolytic cascade is suppressed by the serine protease inhibitor, endothelial plasminogen activator inhibitor type 1 (PAI1). The PAI1/uPA/uPAR assays report chemical effects on the system (up or down) across diverse cellular platforms: 4H, 3C, CASM3C, and hDFCGF noted above; BE3C (human bronchial epithelial cells stimulated with IL-1β + TNFα + IFNϒ); and KF3T (human keratinocytes + fibroblasts stimulated with IL-1β + TNFα + IFNϒ + TGF-β). The pVDC signature has features for thrombomodulin (THBD) and the thromboxane A2 (TBXA2) receptor that

participate in the regulation of endothelial migration during angiogenic sprouting. THBD is a type I transmembrane glycoprotein that mediates regulator of uPA/uPAR and TBXA2 is an angiogenic eicosanoid generated by endothelial cyclooxygenase-2 (COX-2) following VEGF- or bFGF stimulation. THBD protein expression was monitored in the 3C and CASM3C BioMAP systems (up, down) and TBXA2 was assayed for ligand binding in the NovaScreen platform.

Endothelial cell migration and proliferation: the pVDC signature includes assays for human primary vascular cultures (endothelial and vascular smooth muscle cells). Assays for nuclear localization of beta-catenin (CTNB) are based on the principle that nuclear translocation activates pathways important for endothelial cell migration, proliferation and survival during capillary network formation in HUVEC cells [Muller et al. 2002; Masckauchan et al. 2005].

Vascular stabilization: The signature has features for transforming growth factor-beta 1 (TGF-b), which regulates vascular morphogenesis and integrity, and for Tie2 - a receptor tyrosine kinase activated by the angiopoietins (ANG1, ANG2) that function stabilize nascent vasculature. The pVDC signature has features for the anti-angiogenic phosphatases PTEN (phosphatase and tensin homolog), PTPN11 (tyrosine-protein phosphatase non-receptor type 11) and PTPN12, and endothelial-specific receptor tyrosine protein phosphatase beta (PTPRB). Matrix metalloproteinases (MMPs) 1/2/9 aggregate features on biochemical activity and cellular function of zinc-dependent endopeptidases MMP1, MMP2 and MMP9 that facilitate angiogenesis through ECM degradation by activated endothelial cells.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	305	Inhibition, VegfR2	Inhibition, VegfR2
2	KE	28	Reduction, Angiogenesis	Reduction, Angiogenesis
3	KE	110	Impairment, Endothelial network	Impairment, Endothelial network
4	KE	298	Insufficiency, Vascular	Insufficiency, Vascular
5	AO	1001	Increased, Developmental Defects	Increased, Developmental Defects

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, VegfR2	adjacent	Reduction, Angiogenesis	High	High
Reduction, Angiogenesis	adjacent	Impairment, Endothelial network	High	Moderate
Impairment, Endothelial network	non-adjacent	Insufficiency, Vascular	Moderate	Low
Insufficiency, Vascular	non-adjacent	Increased, Developmental Defects	High	Moderate

Stressors

Name	Evidence
Vatalanib	
Sunitinib malate Sunitinib (INN)	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
Conception to < Fetal	High
Pregnancy	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
mouse	Mus musculus	High	NCBI
rats	Rattus norvegicus	Low	NCBI
zebrafish	Danio rerio	High	NCBI

Toxicity testing in the 21st century is moving toward using high-throughput screening assays to rapidly test thousands of chemicals against hundreds of molecular targets and biological pathways, and to provide mechanistic information on chemical effects in human cells and small model organisms. First-generation predictive models for prenatal developmental toxicity have revealed a complex web of biological processes with many connections to vasculogenesis and angiogenesis. Disruption of embryonic vascular development as a potential adverse outcome pathway (AOP) framework leading to developmental toxicity. Further evidence comes from an analysis of pharma compounds to which women of reproductive age were exposed, leading to the implication of vascular disruption as one of six potential mechanisms of teratogenesis. We reviewed embryonic vascular development and important signals for vascular development (local growth factors and cytokines such as VEGF-A and TGF-beta, components in the plasminogen activator system, and chemotactic chemokines). Genetic studies have shown that perturbing these signals can lead to varying degrees of adverse consequences, ranging from congenital angiodysplasia to fetal malformations and embryolethality. The molecular targets and cellular behaviors required for vascular development, stabilization and remodeling are amenable to in vitro evaluation. Evidence for chemical disruption of these processes is available for thalidomide, estrogens, endothelins, dioxin, retinoids, cigarette smoke, and metals among other compounds. Although not all compounds with developmental toxicity show an in vitro vascular bioactivity signature, many 'putative vascular disruptor compounds' invoke adverse developmental consequences. As such, an adverse outcome pathway perspective of embryonic vascular development can help identify useful information for assessing adverse outcomes relevant to risk assessment and efficient use of resources for validation.

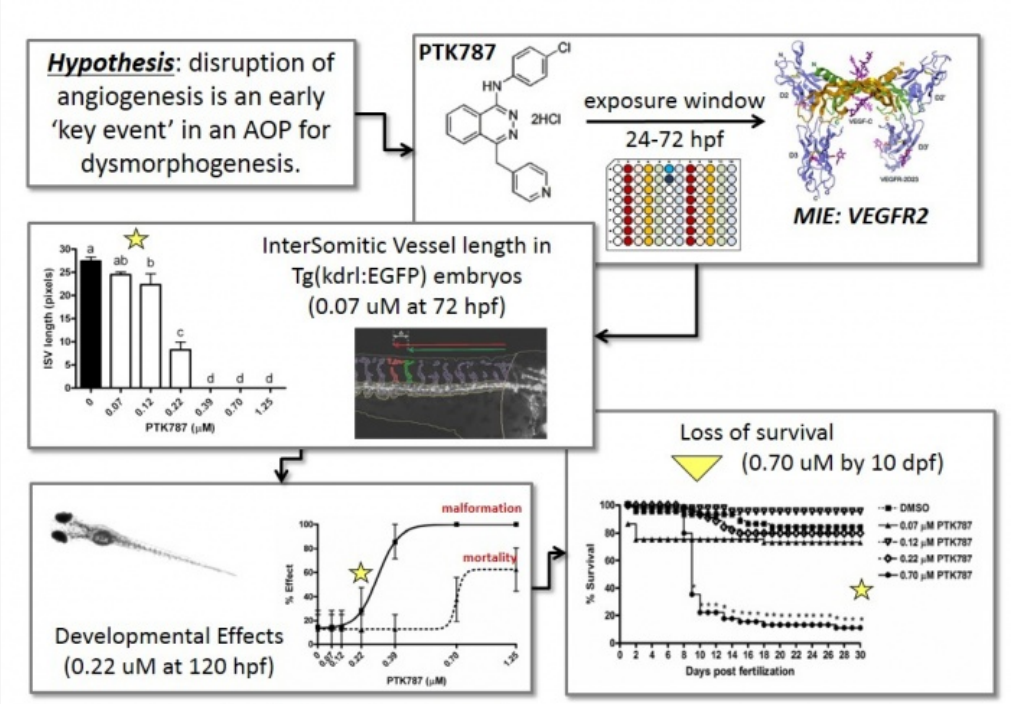
Essentiality of the Key Events

We devised an AOP framework for embryonic vascular disruption based on evidence from the open literature and public databases [Knudsen and Kleinstreuer, 2011]. Cellular behaviors linked to molecular targets in angiogenesis, and to some extent vasculogenesis, are well-described in the literature. Critical cell types include: angioblasts (AB) as direct precursors to primary endothelial cells; endothelial ‘tip’ cells (EC-tip) mediate angiogenic sprouting; endothelial ‘stalk’ cells (EC-stalk) proliferate in the wake of an angiogenic sprout; macrophage cells (MCs) release cytokines, chemokines, and growth factors; and stromal cells (SCs) are recruited to the nascent vascular wall for vessel stabilization. As such, cellular consequences vary spatiotemporally and may be defined by the VEGF-gradient and VEGF-response. Setting up VEGF gradients is a multicellular phenomenon, determined by VEGF expression and processing (eg, MCs, SCs) and biochemical corridors set up by the extracellular matrix and the VEGFR1 decoy receptor (eg, EC-stalk). EC-tip is the critical VEGFR2-responsive cell type displaying exploratory and migratory behavior during angiogenesis. Arsenic, for example, was shown to disrupt these behaviors [Shirinifard et al., 2013]. The impact of chemicals on distinct cellular behaviors or the signaling networks can be studied in vitro utilizing pluripotent stem cells, endothelial tubulogenesis assays, and aortic explants [Sarkanen et al. 2010;Kleinstreuer et al. 2013; Tal et al. 2014]. Downstream consequences of vascular disruption can be tracked in more integrated embryonic systems including rodent whole embryo culture and transgenic endothelial zebrafish reporter lines (e.g., VEGF/Rs). Disruption of vasculogenesis or angiogenesis can adversely impact the embryo in many ways, leading to intrauterine growth retardation (IUGR), skeletal malformations, functional deficits and neonatal death. Blood vessel formation is necessary for the uterine cycle and placentation and, therefore, can affect female fertility leading to implantation failure, pregnancy loss, preeclampsia and preterm labor. This AOP will focus on embryonic development where there is sufficient information on the necessity of vascular development for normal development (e.g., appendicular and axial structures) as well as a direct link between in utero vascular disruption and limb and other defects in humans [Husain et al. 2008; Gold et al. 2011].

Weight of Evidence Summary

Weight of evidence for the MIE and AO are strong; the intermediate KEs have in some cases strong evidence but in other cases weaker evidence, due to the lack of quantitative information. the KERs are biologically plausible. Several manuscripts have been published recently that bolster weight of evidence [Belair et al. 2016; Nguyen et al. 2017; Tal et al. 2017; McCollum et al. 2017; Ellis-Hutchings et al. 2017; Saili et al. 2019; Zurlinden et al. 2020].

Quantitative Consideration



1 *SOURCE: Tal et al. (2014) Reprod Toxicol (in press).*

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Appendix 1

List of MIEs in this AOP

Event: 305: Inhibition, VegfR2

Short Name: Inhibition, VegfR2

Key Event Component

Process	Object	Action
vascular endothelial growth factor receptor 2 binding	vascular endothelial growth factor receptor 2	decreased
vascular endothelial growth factor receptor 2 binding	vascular endothelial growth factor receptor 1	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects	MolecularInitiatingEvent

Stressors

Name
Vatalanib
Sunitinib malate Sunitinib (INN)

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

somatic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Chemical effects on VEGF-A binding to VEGFR2 has been demonstrated for 6 different inhibitors using recombinant VEGF-A(165) [Gustafsdottir et al. 2008]. Among the inhibitors were DNA/RNA aptamers, neutralizing antibodies directed against VEGF-A or VEGFR2, recombinant competitive protein, and a low molecular weight synthetic molecule. A pharmacological panel of small molecule inhibitors of VEGFR inhibitors is known, having varied activities on VEGFR2 and other members of the same receptor tyrosine kinase family as the VEGF receptors, including the platelet-derived growth factor receptor β (PDGFR-β). These compounds include Vatalanib (VEGFR2/PDGFRβ/c-kit inhibitor), Sunitinib (VEGFR1/VEGFR2/PDGFR inhibitor), and Semaxinib (VEGFR2 inhibitor).

Vatalanib, also known by the code name PTK787, is a potent vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor that inhibits VEGFR2/KDR and VEGFR1/Flt-1 with the half maximal inhibition concentration IC50 values of 0.037 µM and 0.077 µM, respectively [Wood et al. 2000]. It also inhibits to a lesser degree PDGFR-β. Liganding VEGFR2 leads to receptor dimerization and autophosphorylation on tyrosine residues, which initiates signal transduction [Kendall et al. 1999]. Using a double antibody chemiluminescence assay, PTK787 was shown to block VEGF-induced auto-phosphorylation of VEGFR2 with an IC50 of 0.017 µM in human endothelial cells (HUVECs) and concentration-dependent suppression of endothelial migration and tumorigenic formation of microvessels [Wood et al. 2000].

Vatalanib

Evidence that this VEGFR2 inhibition can be chemically initiated with impacts on embryogenesis, transgenic TG(flk1:GFP) zebrafish embryos were used to visualize and quantify blood vessel formation [Tal et al. 2014]. The embryos were exposed to Vatalanib at concentrations ranging from 0.07-1.25 uM during the period from 24- to 72 hours post fertilization (hpf). An evaluation of blood vessel development and developmental toxicity showed clear evidence for concentration-dependent disruption, and a comparison of the VEGFR2 inhibitor (PTK787) with an EGFR inhibitor (AG1478) showed regional specificity for adverse effects on vascular patterning and gross morphology. This specificity provides evidence for chemical initiation of VEGFR2 inhibition in the embryo.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra fish	Danio rerio	Moderate	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Low	NCBI
human	Homo sapiens	Moderate	NCBI

There is strong phylogenetic conservation of VEGFR2 genes [Shibuya, 2002]. For example, the amino acid homology ranges from 79.9 - 96.1% for the critical autophosphorylation domain across species of fish, birds, rodents with humans. This suggests a conserved molecular basis to regulation of blood vessel development and implies broad taxonomic applicability to VEGFR2 inhibition. Direct evidence for this comes from the susceptibility of vascular development to pharmacological inhibitors of human VEGFR2 kinase activity. Vatalanib (PTK787), for example, is a potent inhibitor of human VEGFR2 kinase activity [Wood et al. 2002] and disrupted angiogenic vessel formation in early zebrafish embryos at submicromolar concentrations [Tal et al. 2014].

Key Event Description

The VEGF-VEGFR system is an important molecular regulator of various processes linked to physiological and pathological blood

vessel development, from early embryonic to adult stages. The central players in this system among vertebrate species are three vascular endothelial growth factor receptors (VEGFR1, VEGFR2, VEGFR3) and five VEGF family members that bind and activate these various receptors during vasculogenesis, angiogenesis and lymphogenesis [Shibuya, 2013]. A search of the PubMed literature for these Medical Subject Heading (MeSH) terms returned 41,371 entries 1989-2014 (December 4, 2014); 4982 of those entries had broad focus on ‘vascular’ and ‘development’ headings.

Vascular endothelial growth factor-A (VEGF-A), in particular the VEGF165 splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. This is evidenced by immature blood vessel formation and embryonic lethality in mutant mouse embryos heterozygous for the Vegfa-null allele [Ferrara et al. 1996; Carmellet et al. 1996]. VEGF-A is a soluble protein that acts directly on endothelial cells through two receptor tyrosine kinases: VEGFR1 (Flt-1) and VEGFR2 (KDR). The former is a decoy receptor that traps VEGF-A into corridors preventing interaction with the active receptor, VEGFR2. When liganded, VEGFR2 induces endothelial tip cell proliferation, survival, and vascular permeability. Various factors leading to a change in VEGF-A gradients will be considered. This could include a change in the local production of VEGF-A, an increase in the decoy receptor (VEGFR1), or a drop in the expression or activity of VEGFR2. Chemical effects may commence at VEGF receptors (VEGFRs) by influencing local VEGF-A ligand production, ligand binding, receptor tyrosine kinase activity, or crosstalk with angiogenic chemokines, cytokines and growth factors. VEGF-A is locally produced in the vicinity of target endothelial cells. Hypoxia (and chemical hypoxia) increases VEGF-A production through the HIF-alpha transcription pathway. VEGF-A can be trapped in the extracellular milieu by binding to components in the extracellular matrix (ECM) or VEGFR1. Since VEGFR1 binds VEGF-A with 10-fold greater affinity than does VEGFR2, VEGF-A is liberated by ECM breakdown during morphogenetic remodeling of tissues or matrix metalloproteinase (MMP) production during chemical injury. These events can positively or negatively regulate the local bioavailability of VEGF-A to its cognate receptor.

Targeted disruption of VEGFR1 or VEGFR2 is early embryonic lethal; however, the vascular phenotypes differ in either case. VEGFR1-mutant (Flt1-null) embryos display excessive endothelial cell growth leading to disorganization of the vascular network [Fong et al. 1995] whereas VEGFR2-mutant (Flk1-null) embryos die from a lack blood vessel network formation [Shalaby et al. 1995]. This duality is relevant to the VEGFR disruption because receptor affinity for VEGF is ten-fold higher at VEGFR1 but kinase activity is ten-fold higher at VEGFR2 [Fischer et al. 2008; Shibuya, 2013]. As such, VEGFR2 promotes angiogenesis whereas VEGFR1 acts as a ligand-trap to prevent VEGF-A interaction with VEGFR2 [Hiratsuka et al. 1998]. However, VEGFR2 activation is considered the master switch of developmental angiogenesis. In addition to upstream effects on VEGF-A production or trapping, the inhibition of VEGFR2 can be engaged by interference interference with VEGF-A binding to VEGFR2, inhibition of receptor tyrosine kinase activity, or reduced expression of the VEGFR2 protein. Given the complex nature of the VEGF-VEGFR system, disruption of VEGF2 has diverse origins.

How it is Measured or Detected

A structure-activity relationship (SAR) analysis of a 73,000-compound library using an HIF-1a: VEGF secretion assay identified 350 actives for followup [Xia et al. 2009]. Proximity Ligation Assays have been used to identify small molecule inhibitors of VEGF-A binding to its receptors [Gustafsdottir et al. 2008]. This assay is: fit for the purpose of monitoring the formation and inhibition of VEGF-A–receptor complexes; defines chemical disruption of VEGF-A direct binding to its receptors (VEGFR1, VEGFR2); correlates well with results obtained by measuring receptor phosphorylation (VEGFR2); and allows evaluation of the half-maximal inhibitory concentration (AC50) from a concentration-response curve.

VEGFR2 inhibition can be detected by measures of capacity (receptor density, expression levels) and bioactivity (tyrosine kinase activity) utilizing molecular probes and pharmacological reagents. As part of a broader AOP framework for vascular developmental toxicity, VEGFR2 inhibition is anchored to genetic models having strong phenotypic evidence for adverse developmental outcomes. To organize what is known, an AngioKB knowledgebase was built from high-throughput PubMed queries utilizing Medical Subject Heading (MeSH) terms for vasculogenesis and angiogenesis in the embryo, fetus or development [Knudsen and Kleinstreuer, 2011]. These unstructured data were supplemented with information culled from the MGI Mammalian Phenotype Ontology (MPO) Browser (http://www.informatics.jax.org, accessed October 31, 2011) for 'abnormal vasculogenesis' (MP:0001622, aberrant process of the initial establishment of the vascular network; 78 annotations) and 'abnormal angiogenesis' (MP:0000260, aberrant process of blood vessel formation and the subsequent remodeling process - does not refer to the initial establishment of the vascular network; 892 annotations). The relevant genes were then mapped to the ToxCast assay portfolio [1]. This yielded an overlap of 25 different sectors for pathways in blood vessel development [Knudsen and Kleinstreuer 2011], including the VEGF-VEGFR system covering many diverse studies in the open literature. The predictive signature for vascular disruption (pVDCs) incorporates several assays that directly measure VEGFR2 capacity (BSK_4H_VEGFRII_down and BSK_4H_VEGFRII_up) and bioactivity (NVS_ENZ_hVEGFR1 and NVS_ENZ_hVEGFR2). Importantly, VEGFR2 is inactive when non-liganded so measures of VEGFR2 capacity in the BioSeek (BSK) human 4H primary endothelial cell assay can register an increase or decrease in capacity depending on whether the VEGFR2 inhibition is being detected by a change in VEGFR2 protein levels [Kleinstreuer et al. 2014]. Liganding VEGFR2 leads to receptor dimerization and autophosphorylation on tyrosine residues, which initiates signal transduction [Kendall et al. 1999]. This bioactivity is measured by a cell-free assay for human recombinant VEGFR2 that can register a concentration-dependent activation or inhibition [Knudsen et al. 2009; Sipes et al. 2011].

Analysis of VEGFR2 expression under different physiological and toxicological contexts can be determined by standard array-based assays to define regulation of the angiogenic transcriptome, as well as targeted non-array methods to characterize cell-specific profiles during in vivo and in vitro development [Dumont et al. 1995; Abbott et al. 2000; Drake et al. 2007; Murakami et al. 2011]. In addition, the ontogenetic profile of VEGFR1 and VEGFR2 expression can be mapped in reporter zebrafish embryos under specific control of Flk or Flt gene regulatory elements [Tal et al. 2014]. Zebrafish possess 72 orthologs for 70% of human genes and 86% of 1318 human drug targets. Transgenic zebrafish that express reporter gene in developing blood vessels are fit for purpose to: visualize blood vessel formation during early development; localize and quantitate regional effects of chemicals on a phenotypic

readout of angiogenic vessel formation; assess the reproducibility of vascular disruption across species; and evaluate the half-maximal inhibitory concentration (AC50) from a concentration-response curve.

Downstream consequences to signal transduction can be measured by specific targets of VEGFR2 tyrosine kinase activity. Various examples of bioassays that measure the growth of blood vessels and the effects of specific inhibitors include in vitro assays of endothelial cell migration and proliferation. Some assays test human endothelial cells in primary culture models (e.g., HUVEC), stem-cell derived systems that are capable of de novo assembly into capillary networks, or genetically engineered mouse and zebrafish embryo, and computational models [Mueller et al. 2000; Dorrell et al. 2002; Xia et al. 2009; Chapell et al. 2013; Kleinstreuer et al. 2013; Shirinifard et al. 2013]. These assays and models are: fit for the purpose of defining optimal VEGF-A levels for angiogenesis; screening large inventories of small molecules for VEGF-A secretion over a range of chemical concentrations and low oxygen tension; linkage of VEGFR2 inhibition with the physiological initiating event; and evaluation of the half-maximal inhibitory concentration (AC50) from a concentration-response curve.

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List of Key Events in the AOP

Event: 28: Reduction, Angiogenesis

Short Name: Reduction, Angiogenesis

Key Event Component

Process	Object	Action
angiogenesis		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
stromal cell

Domain of Applicability

ToxCast high-throughput screening (HTS) data for 25 assays mapping to targets in embryonic vascular disruption signature [Knudsen and Kleinstreuer, 2011] were used to rank-order 1060 chemicals for their potential to disrupt vascular development. The predictivity of this signature is being evaluated in various angiogenesis assays, including angiogenic sprouting in human endothelial cells [Belair et al. 2016] and trangenic zebrafish embryos [Tal et al. 2016].

Belair et al. [2016] designed and characterized a chemically human angiogenesis pPSC-EC sprouting model that responded appropriately to several reference pharmacological angiogenesis inhibitors, including Vatalanib/PTK787, which suggests the functional role of VEGFR2. Several pVDCs from the ToxCast library also inhibited angiogenic sprouting in this assay. Because gene sequence similarity of the ToxCast pVDC signature is comprised of proteins that primarily map to human in vitro and biochemical assays, the U.S. EPA SeqAPASS tool was used to assess the degree of conservation of signature targets between zebrafish and human, as well as other commonly used model organisms in human health and environmental toxicology research [Tal et al. 2017]. This approach revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species. Homology appeared first in the receptor tyrosine kinase signaling systems, followed in turn by the urokinase plasminogen activating (uPA) receptor (uPAR) system and chemokine/G-protein coupled receptor system.

Key Event Description

Developmental angiogenesis involves a complex interplay between the native extracellular matrix, vascular endothelial cells (EC), growth factors, and cytokines/chemokines [Knudsen and Kleinstreuer, 2011].

How it is Measured or Detected

In vitro models are presently used to study EC function and screen for angiogenesis inhibitors based on effects on cell proliferation, sprouting behavior, and tubulogenesis. Sprouting is driven by matrix metalloproteinase (MMP) activity whereas tubulogenesis shows MMP-dependence only in three-dimensional (3D) contexts. Furthermore, the VEGF-dependence is unclear in some tubulogenesis assay platforms and this further limits comparisons of EC tubulogenesis to VEGF-dependent vascular formation in vivo. As such, EC sprouting models that recapitulate MMP-dependent and VEGF-dependent endothelial cell invasion provide a more physiologic context for modeling early stages of angiogenesis and can be used to evaluate the extracellular matrix (ECM) degradation and invasion characteristic of angiogenic sprouting in vivo [Belair et al. 2016].

Functional assays used to evaluate angiogenic sprouting utilize natural (ECM) and synthetic (hydrogel) matrices that support growth factor-dependent endothelial cell proliferation, migration and invasive behaviors. EC sprouting models that recapitulate matrix metalloproteinase-dependent and VEGF-dependent endothelial cell invasion provide a physiologic context for modeling early stages of angiogenesis. Endothelial cell migration is directed by chemotactic, haptotactic, and mechanotactic stimuli and degradation of the ECM to enable progression of the migrating cells. It requires the activation of several signaling pathways that converge on cytoskeletal remodeling and follows a molecular cascade in which the endothelial cells extend filopodial processes and progress forward [Lamalice et al. 2007]. Pro-angiogenic signals, such as VEGF-A, together with Notch signaling controls whether specific endothelial cells become leading 'tip cells' or trailing 'stalk cells'. Angiogenic sprouts then convert into endothelial tubules and form connections with other vessels, which requires the local suppression of motility and the formation of new cell-cell junctions [Eilken and Adams, 2010]. A unique method to encapsulate endothelial cells at a controlled cell density in hydrogel spheres surrounded by a synthetic ECM allows for quantitative analysis of EC sprouting for enhanced-throughput screening in a chemically-defined sprouting model [Belair et al. 2016]. Another approach to detecting effects on angiogenic sprouting dynamics is live-cell imaging in transgenic zebrafish embryos [Shirinfard et al. 2013].

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Event: 110: Impairment, Endothelial network

Short Name: Impairment, Endothelial network

Key Event Component

Process	Object	Action
endothelium development		abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects	KeyEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Organ term

Organ term

embryo

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Blood vessel development utilizes highly conserved molecular pathways that are active across vertebrate species. Anatomically, however, the molecular toolbox for vasculogenesis/angiogenesis has varied themes for arterial, venous, and lymphatic channels, as well as across different organs and species [Tal et al. 2017]. ToxCast high-throughput screening (HTS) data for 25 assays mapping to targets in embryonic vascular disruption signature [Knudsen and Kleinstreuer, 2011] were used to rank-order 1060 chemicals for their potential to disrupt vascular development. The predictivity of this signature is being evaluated in various angiogenesis assays, including tubulogenesis in endothelial cells from zebrafish, chick, mouse and human species [Tal et al. 2017; Vargesson et al. 2003; Saili et al. 2019; McCollum et al. 2017; Nguyen et al. 2017, Zurlinden et al. 2020]. As an example, a zebrafish embryo vascular model in conjunction with a mouse endothelial cell model identified 28 potential vascular disruptor compounds (pVDCs) from ToxCast. These exposures invoked a plethora of vascular perturbations in the zebrafish embryo, including malformed intersegmental vessels, uncondensed caudal vein plexus, hemorrhages and cardiac edema; 22 pVDCs inhibited endothelial tubulogenesis in an yolk-sac-derived endothelial cell line [McCollum et al. 2017]. The VEGF pathway was implicated across mouse-zebrafish species. Because gene sequence similarity of the ToxCast pVDC signature is comprised of proteins that primarily map to human in vitro and biochemical assays, the U.S. EPA SeqAPASS tool was used to assess the degree of conservation of signature targets between zebrafish and human, as well as other commonly used model organisms in human health and environmental toxicology research [Tal et al. 2017]. This approach revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species.

Key Event Description

In embryological terms the angiogenic cycle entails a stepwise progression of de novo blood vessel morphogenesis (vasculogenesis), maturation and expansion (angiogenesis), and remodeling [Hanahan, 1997; Chung and Ferrara 2011; Coultas et al. 2005]. These events commence as angioblasts migrate, proliferate, and assemble into a tubular network. With maturation, the endothelial tubules co-opt local stromal cells as pericytes and smooth muscle. Local signals acting on receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and glycosyl phosphatidyl-inositol (GPI)-anchored receptors, and later vascular flow-mediated signals. The process of endothelial assembly into a tubular network may be disrupted by environmental agents [Sarkanen et al. 2010; McCollum et al. 2017; Saili et al. 2019; Nguyen et al. 2016; Tal et al. 2017].

How it is Measured or Detected

Endothelial tubule formation (tubulogenesis) can be monitored both qualitatively and quantitatively in vitro using different human cell-based angiogenesis assays that score endothelial cell migration and the degree of tubular network formation, including cell counts, tubule counts, tubule length, tubule area, tubule intensity, and node counts [Muller et al. 2002; Masckauchan et al. 2005; Sarkanen et al. 2010; Knudsen et al. 2016; Nguyen et al. 2016]. Standard practice for reproducible in vitro tubule formation uses endothelial cells co-cultured with stromal cells [Bishop et al. 1999]. Cell types commonly employed are human umbilical endothelial cells (HUVECs) or more recently induced pluripotent stem cells (iPSCs) derived to endothelial cells through various differentiation and purification protocols. The assay is run in agonist or antagonist modes to detect chemical enhancement or suppression of tubulogenesis. Synthetic hydrogels are shown to promote robust in vitro network formation by HUVEC or iPSC-ECs as well as their utilization to detect putative vascular disruptive compounds [Nguyen et al. 2017]. Endothelial networks formed on synthetic hydrogels showed superior sensitivity and reproducibility when compared to endothelial networks formed on Matrigel.

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Event: 298: Insufficiency, Vascular

Short Name: Insufficiency, Vascular

Key Event Component

Process	Object	Action
blood circulation	blood	decreased
	capillary plexus	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Organ term

Organ term

embryo

Domain of Applicability

Complex functional assays such as the rat aortic explant assay (AEA), rat whole embryo culture (WEC), and the zebrafish embryotoxicity (ZET) along with transcriptomic signatures provide a tiered approach to evaluate HTS signatures and their taxonomic implications for conserved pathways to prioritize further in vivo testing studies [Ellis-Hutchings et al. 2017].

Key Event Description

The cardiovascular system is the first functional organ system to develop in the vertebrate embryo, reflecting its critical role during normal development and pregnancy. Blood vessels are key regulators of organogenesis by providing oxygen, nutrients and molecular signals [Maltepe et al. 1997; Chung and Ferrara, 2011; Eshkar-Oren et al. 2015].

Vascular development commences in the early embryo with in situ formation of nascent vessels from angioblasts, leading to a primary capillary plexus (vasculogenesis). After the onset of blood circulation, the primary vascular pattern is further expanded as new vessels sprout from pre-existing vessels (angiogenesis). Both processes, vasculogenesis and angiogenesis, are regulated by genetic signals and environmental factors dependent on anatomical region, physiological state, and developmental stage of the embryo. The developing vascular network is further shaped into a hierarchical system of arteries and veins, through progressive effects on blood vessel arborization, branching, and pruning (angioadaptation). These latter influences include hemodynamic forces, regional changes in blood flow, local metabolic demands and growth factor signals.

Disruptions in embryonic vascular patterning-adaptation may result in adverse pregnancy outcomes, including birth defects, angiodysplasias and cardiovascular disease, intrauterine growth restriction or prenatal death. Some chemicals may act as potential vascular disrupting compounds (pVDCs) altering the expression, activity or function of molecular signals regulating blood vessel development and remodeling. Critical pathways involve receptor tyrosine kinases (e.g., growth factor-signaling), G-protein coupled receptors (e.g., chemokine signaling), and GPI-anchored receptors (e.g. uPAR system). Embryonic vascular disruption has been implicated in the etiology of human birth defects associated with medications taken by women of child-bearing potential (WOCBP) [van Gelder et al. 2009] and thalidomide teratogenesis in animal studies [Therapontos et al. 2009; Vargesson et al. 2015].

How it is Measured or Detected

A number of experimental and computational models are fit for purpose of monitoring vascular development and assessing vascular insufficiency [Knudsen and Kleinstreuer 2011]. These include: transgenic zebrafish that express enhanced green fluorescent protein in blood vessels [Jin et al. 2005]; chick embryos [Therapontos et al. 2009; Vargesson, 2015]; and rodent embryo culture [Ellis-Hutchings et al. 2016]. Phenotypic readouts of angiogenic vessel formation of the intersegmental vessels (ISVs) in transgenic zebrafish embryos has been used to screen and validate anti-angiogenic compounds [Tran et al. 2007; Yano et al. 2012; Yozzo et al. 2013; Tal et al. 2014; McCollum et al. 2017]. In transgenic zebrafish embryos, live-cell imaging has been used to quantitatively detect the trajectory dynamics of vascular patterning [Clendenon et al. 2013; Shirinfard et al. 2013] and confocal cell imaging has been used to develop a quantitative assay capable of detecting relatively subtle changes (~8%) in ISV length relative to controls during chemical exposure [Tal et al. 2017]. Computational approaches have also been used to predict vascular insufficiency. For example, an in vitro signature for potential vascular disrupting chemicals (pVDCs) was mined for developmental toxicity based on ToxCast [Kleinstreuer et al. 2011; Knudsen and Kleinstreuer, 2011]. This has since been applied to the ToxCast inventory to rank order 1060 chemicals for validation testing [McCollum et al. 2017; Tal et al. 2017; Saili et al. 2019. As such, a chemical's potential to disrupt vascular patterning, remodeling, or utero-placental circulation could be a class predictor of developmental toxicity solely based on HTS in vitro data in combination with our understanding the embryology behind vascular development.

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List of Adverse Outcomes in this AOP

Event: 1001: Increased, Developmental Defects

Short Name: Increased, Developmental Defects

Key Event Component

Process	Object	Action
anatomical structure morphogenesis		morphological change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects	AdverseOutcome

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Wilson's Principles of Teratology (circa 1977) support the taxonomic applicability of teratogenesis. According to these long-standing Wilson's principles, the first on "Susceptibility to Teratogenesis Depends on the Genotype of the Conceptus and a Manner in which this Interacts with Adverse Environmental Factors". This principle has four main tenets:

i) species differences account for the fact that certain species respond to particular teratogens where others do not, or at least not to the same extent (e.g., humans and other primates are vulnerable to thalidomide induced phocomelia whereas rodents are not);

ii) strain and intralitter differences account for the fact that some lineages of the same species with different genetic backgrounds can differ in teratogenic susceptibility;

iii) gene-environment interplay results in different patterns of abnormalities between organisms with the same genome raised in different environments, and between organisms with different genomes raised in the same environment; and

iv) multifactorial causation accounts for the complex interactions involving more than one gene and/or more than one environmental factor.

Key Event Description

The risks for chemical effects on the reproductive cycle are broadly defined in two categories for regulatory purposes: reproductive (fertility, parturition, lactation) and developmental (mortality, malformations, growth and functional deficits). With respect to apical endpoints for developmental defects, the International Conference on Harmonization regulatory guidelines for embryo-fetal developmental toxicity testing (ICH 2005) require studies in both a rodent and a non-rodent species, usually rat and rabbit. The current paradigm was developed in response to the pandemic of phocomelia associated with maternal exposure to thalidomide during early pregnancy [Schardein 2000]; however, dose ranges of thalidomide that were teratogenic in the rabbit induced embryo-fetal loss in the rat [Janer et al. 2008]. This observation is consistent with current knowledge that the specificity of the manifestations of embryo-fetal toxicity may vary greatly between species, and even between strains within the same species [Hurtt et al. 2003; Janer et al. 2008; Knudsen et al. 2009; Rorije et al. 2012; Theunissen et al. 2016]. Recent advances in our knowledge of the molecular and cellular bases of embryogenesis serve to provide a deeper understanding of the fundamental developmental mechanisms that underlie Wilson's Principles of Teratology as the standard formulation of the basic tenets of the field [Friedman 2010].

Developmental defects includes the four main types of defects observed in regulatory guideline studies (prenatal loss, malformations, low birth weight, and postnatal function). Any or all of these developmental defects may occur within the same litter or study. Congenital malformations refer to alterations in normal development that result from intrinsic (genetically programmed) or extrinsic (environmentally induced) perturbations of development. Mechanistically, some pathways may lead to specific types of malformations; however, a fundamental principle is that all four types of endpoints are important for hazard assessment. This is because even a simple MIE may disrupt the embryo in multiple ways that depend on the nature of the insult and timing of exposure.

How it is Measured or Detected

Developmental defects are typically assessed by observational studies of animal models and by human epidemiological studies. For animal models, the apical endpoints typically derive from a litter-based evaluation of fetuses just prior to birth or beyond. A study design fit for the purpose of regulatory toxicology adheres to regulatory guidelines specified by OECD Test Guideline No. 414 (Prenatal Developmental Toxicity Study). Prenatal animal studies in mammalian species where exposure to a drug or chemical is administered to the dam describe the occurrence and severity of effects to the mother and fetuses and perform statistical evaluations on a litter basis since the dam is the exposure unit. Latent effects that do not manifest at term, or are not reliably diagnosed until postnatal development or subsequent generations, may be detected by OECD Test No. 415 (One-Generation Reproduction Toxicity Study) or Test No. 416 (Two-Generation Reproduction Toxicity).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 335: Inhibition, VegfR2 leads to Reduction, Angiogenesis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Studies have demonstrated a quantitative relationship between VEGFR2 signaling and angiogenic sprouting dynamics in human endothelial cells [Belair et al. 2016] and zebrafish embryos [Shirinifard et al. 2013].

Key Event Relationship Description

VEGFR2 is the master switch of angiogenesis, which is triggered by binding to its cognate ligand (VEGF-A). In the induction of an angiogenic sprout, VEGF-A triggers a response in endothelial cells (EC). VEGF-A has a mitogenic effect on EC 'stalk cells' and promotes exploratory and behavior and motility of the pioneering ‘tip cells’. The latter extend filopodial processes toward the VEGF-A gradient to pioneer sprouting, while stalk cells proliferate to extend the microvessel and lumenize it for blood transport. Angiogenic sprouting is a complex molecular process [Herbert and Stanier 2011]. An early step is tip cell selection. Endothelial cells are normally suppressed in their tip cell behaviors due to lateral inhibition by Notch-Delta. Lateral inhibition is broken when VEGFR2 is activated by VEGF-A by an uncertain mechanism. Next, EC tip cells extend filopodial processes and migrate along VEGF-A corridors. Their branching to a tree-like network is patterned by the EphrinB2–EPHB4 cell adhesion system. Proliferating EC stalk cells meanwhile follow the pioneering sprout.

Evidence Supporting this KER

Biological Plausibility

VEGFR2 is the most important VEGF-A receptor and is the 'master switch' for angiogenic sprouting [Herbert and Stanier 2011].

Empirical Evidence

Vascular endothelial growth factor-A (VEGF-A), in particular the VEGF165 splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. This is evidenced by immature blood vessel formation and embryonic lethality in mutant mouse embryos heterozygous for the Vegfa-null allele [Ferrara et al. 1996; Carmellet et al. 1996]. Targeted disruption of genes encoding VEGFR1 or VEGFR2 are early embryonic lethal; however, the vascular phenotypes differ in either case. Whereas VEGFR1-mutant (Flt1-null) embryos display excessive endothelial cell growth and disorganization of the vascular network [Fong et al. 1995], VEGFR2-mutant (Flk1-null) embryos die from a lack blood vessel network formation [Shalaby et al. 1995].

Uncertainties and Inconsistencies

Many physiological signals influence VEGF-A production (e.g., hypoxia, estrogen) and post-VEGFR2 signaling. For example, VEGFR2 signals may be influenced by crosstalk with VEGFR1 and VEGFR3, other receptor tyrosine kinases (FGFR, EGFR), G-protein coupled receptors (CXCRs and CCRs), and GPI-linked surface receptors (uPAR) [Kleinstreuer et al. 2011]. The ToxCast pVDC signature includes assays for many of these targets and shows that environmental chemicals perturbing VEGFR2 also affect molecular targets in some other signaling system [Knudsen et al. 2016]. As such, quantitative linkages to VEGF signaling must consider the uncertainties from effects to other MIEs.

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Relationship: 36: Reduction, Angiogenesis leads to Impairment, Endothelial network

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Blood vessel development utilizes highly conserved molecular pathways that are active across vertebrate species. A zebrafish embryo vascular model in conjunction with a mouse endothelial cell model identified 28 potential vascular disruptor compounds (pVDCs) from ToxCast. These exposures invoked a plethora of vascular perturbations in the zebrafish embryo, including malformed intersegmental vessels, uncondensed caudal vein plexus, hemorrhages and cardiac edema; 22 of the also inhibited endothelial endothelial tubulogenesis in an yolk-sac-derived endothelial cell line [McCollum et al. 2016]. The U.S. EPA SeqAPASS tool revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species [Tal et al. 2016].

Key Event Relationship Description

Angiogenic sprouting dynamics drives the complexity and connectivity of endothelial networks in vivo and in vitro. This process is dependent on rates of endothelial tip cell migration and stalk cell proliferation, as well as differential cell adhesion.

Evidence Supporting this KER

Biological Plausibility

Endothelial network formation is dependent on proper regulation of angiogenic sprouting.

Empirical Evidence

Compounds that disrupt angiogenic sprouting behaviors [Belair et al. 2016] also disrupt endothelial tubular network formation [Nguyen et al. 2016].

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List of Non Adjacent Key Event Relationships

Relationship: 125: Impairment, Endothelial network leads to Insufficiency, Vascular

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects	non-adjacent	Moderate	Low

Key Event Relationship Description

In utero vascular disruptions are thought to be associated with a variety of developmental defects [Husain et al. 2008]. Vascular disruption was identified as one of 6 teratogenic mechanisms linked with medications [van Gelder et al. 2010].

Evidence Supporting this KER

Biological Plausibility

In humans, the most common apparent cause of limb deficiencies was found to be vascular disruption defects [Gold et al. 2011]. Many genetic and environmental factors alter molecular pathways regulating angiogenesis [Knudsen and Kleinstreuer, 2011].

Empirical Evidence

Susceptibility to Thalidomide linked to the disruption of immature angiogenic network at time of exposure [Therapontos et al. 2009]. Predicted vascular disrupting chemicals in ToxCast correlate with developmental toxicity [Kleinstreuer et al. 2011].

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Relationship: 1036: Insufficiency, Vascular leads to Increased, Developmental Defects

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects	non-adjacent	High	Moderate

Evidence Supporting this KER

Empirical Evidence

Vascular endothelial growth factor-A (VEGF-A), in particular the VEGF165 splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. This is evidenced by immature blood vessel formation and embryonic lethality in mutant mouse embryos heterozygous for the Vegfa-null allele [Ferrara et al. 1996; Carmellet et al. 1996]. Targeted disruption of VEGFR1 or VEGFR2 is early embryonic lethal; however, the vascular phenotypes differ in either case. VEGFR1-mutant (Flt1-null) embryos display excessive endothelial cell growth leading to disorganization of the vascular network [Fong et al. 1995] whereas VEGFR2-mutant (Flk1-null) embryos die from a lack blood vessel network formation [Shalaby et al. 1995].

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