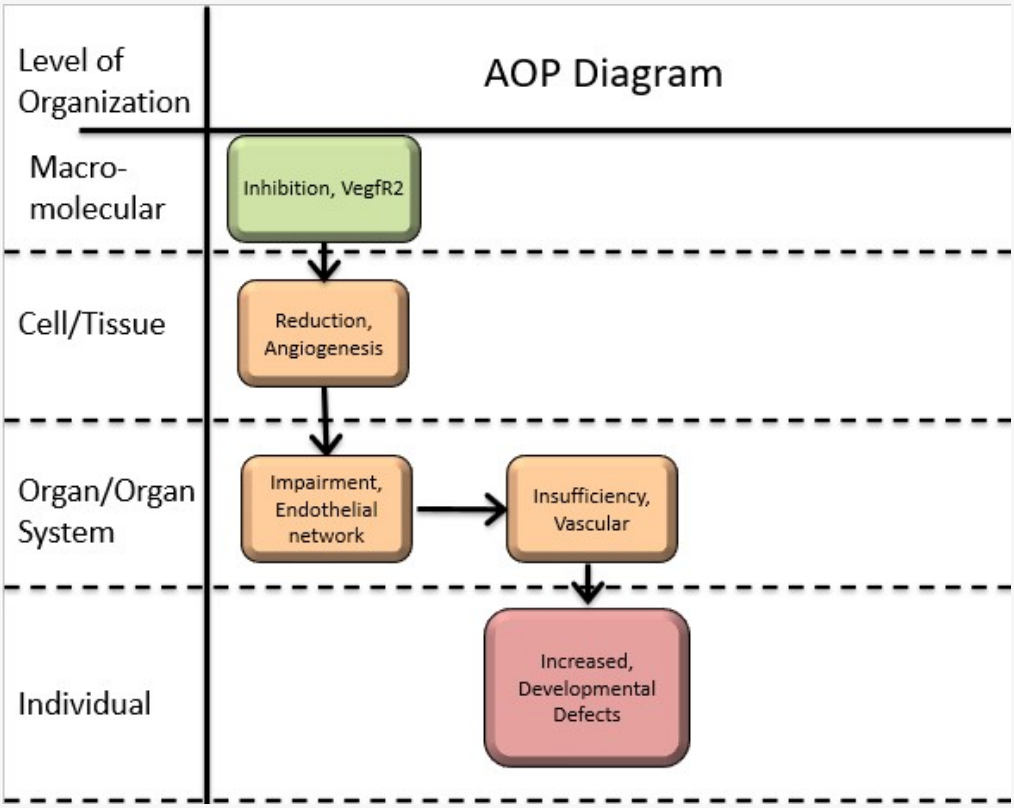
AOP ID and Title:

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

Graphical Representation



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Status

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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

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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

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
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
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

The cardiovascular system is the first organ system to function in the vertebrate embryo, reflecting its critical role during organogenesis [Chan et al. 2002; Jin et al. 2005; Walls et al. 2008]. Blood vessel development commences in the early (sexually undifferentiated) embryo with *de novo* assembly of angioblasts into a primary capillary plexus (vasculogenesis). With 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 developmentally regulated by genetic signals and environmental factors dependent on anatomical region, physiological state, and gestational age of the embryo-fetus [Shalaby et al. 1995; Patan, 2000; Jin et al. 2005; Knudsen and Kleinstreuer, 2011; Eberlein et al. 2021]. Disruption of embryonic vascular development is a potential framework for adverse outcome pathways (AOPs) in developmental toxicity [Knudsen and Kleinstreuer, 2011; Kleinstreuer et al. 2013; Saili et al. 2019; Zurlinden et al. 2020]. Developmental angiogenesis is supported by evidence in genetic models of abnormal vascularization leading to severe developmental phenotypes [Fong et al. 1995; Shalaby et al. 1995; Carmeliet et al. 1996; Maltepe et al. 1997; Abbott and Buckalew, 2000; Chan et al. 2002; Coultas et al. 2005; van den Akker et al. 2007; Eberlein et al. 2021]. This may include cell signals and responses driving formation of the primitive capillary network in the early embryo and extraembryonic membranes (vasculogenesis), the subsequent expansion and patterning of the embryonic and placental vasculature (angiogenesis), and its further stabilization, specialization, and remodeling during growth, organogenesis and differentiation. Additional evidence comes from dysmorphogenesis induced with known anti-angiogenic compounds across multiple vertebrate species (e.g., zebrafish, frog, chick, mouse, rat) [Therapontos et al. 2009; Jang et al. 2009; Rutland et al. 2009; Tal et al. 2014; Vargesson, 2015; Beedie et al. 2016; Ellis-Hutchings et al. 2017; Kotini et al. 2020] and human studies of malformations correlated with genetic and/or environmental factors that target vascular development [Husain et al. 2008; Gold et al. 2011; Vargesson and Hootnick, 2017]. An analysis of pharma compounds to which women of reproductive age were exposed identified vascular disruption as one of six potential mechanisms of teratogenesis [van Gelder et al. 2010]. This AOP is focused on disruption of ‘developmental angiogenesis’ from the perspective of dysmorphogenesis leading to severe developmental defects. Although uterine-decidual vascularization is critically important for healthy pregnancy outcomes, the emphasis here is the direct role on anatomical development of the embryo proper.

Essentiality of the Key Events

Event	Direct Evidence	Indirect Evidence	No experimental evidence	Contradictory experimental evidence
MIE: Inhibition, VegfR2	*****			
KE1: Reduction, Angiogenesis	*****			
KE2: Impairment, Endothelial network	****	*		
KE3: Insufficiency, Vascular	**	***		
AO: Increased, Developmental Defects	***	**		

The Vascular Endothelial Growth Factor (VEGF) pathway is a critical regulatory system for assembly of embryonic blood

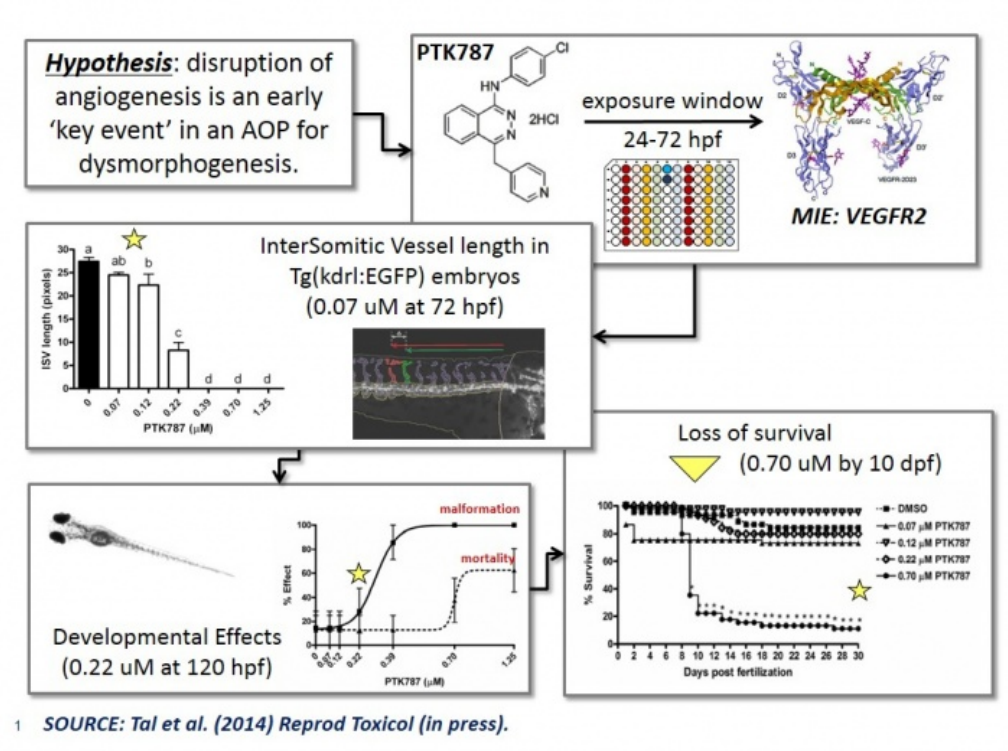
AO: Increased, Developmental Defects				

vessels [Fong et al. 1995; Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara, et al. 1996; Argraves et al. 2002; Hogan et al. 2004; Roberts et al. 2004; Chung and Ferrara, 2011; Shibuya, 2013; Chapell et al. 2016; Jin et al. 2017; Queisser et al. 2021]. The VEGF growth factors belong to the platelet-derived growth factor supergene family. VEGF-A, the major regulator for angiogenesis, binds receptor tyrosine kinases VEGFR-1 (Flt-1) and VEGFR2 (KDR/Flk-1) to regulate endothelial cell proliferation, survival, migration, tubular morphogenesis, and sprouting [Hogan et al. 2004; Douglas et al. 2009; Shibuya, 2013]. This pro-angiogenic effect is locally fashioned as VEGF gradients where the soluble VEGFR1 (sFlt-1) is released from the cell surface as an endogenous VEGF inhibitor that sets up VEGF-A corridors in the developing embryo [Roberts et al. 2004; Chappell et al. 2016]. Genetic studies have shown that perturbing the VEGF signaling system can invoke varying degrees of adverse consequences, ranging from congenital angiodysplasia to fetal malformations and embryolethality [Fong et al. 1995; Ferrara et al. 1996; Eshkar-Oren et al. 2015; Jin et al. 2017]. Filopodial sprouting of the endothelial cell tip (EC-tip) is the critical VEGFR2 responsive cell type specifically in this AOP [Belair et al. 2016a and 2016b]; however, other relevant cell types include: angioblasts (AB) as direct precursors to primary endothelial cells; endothelial 'stalk' cells (EC-stalk) proliferate in the wake of an angiogenic sprout; macrophage/microglial cells (MCs) release cytokines, chemokines, and growth factors; and stromal cells (SCs) are recruited to the nascent vascular wall for vessel stabilization. As such, the VEGF gradient/response system influences a multicellular dimension determined by cellular patterns of VEGF expression and processing (eg, MCs, SCs) and biochemical corridors set up by the extracellular matrix and the VEGFR1 decoy receptor (eg, EC-stalk). Evidence supporting an AOP for chemical disruption is available for thalidomide, estrogens, endothelins, dioxin, retinoids, cigarette smoke, and metals among other compounds [Kleinstreuer et al. 2011; Knudsen and Kleinstreuer, 2011; Shirinifard et al. 2013; Tal et al. 2014 and 2017; McCollum et al. 2017; Toimela et al. 2017; Mahony et al. 2018; Saili et al. 2019; Zurlinden et al. 2020]. Although not all compounds with developmental toxicity show an in vitro vascular bioactivity signature, many 'putative vascular disruptor compounds' (pVDCs) invoke adverse developmental consequences [Kleinstreuer et al. 2011 and 2013]. The molecular and cellular biology of human vascular development, stabilization and remodeling is amenable to *in vitro* assays with human cells [Bishop et al. 1999; Sarkanen et al. 2010; Kleinstreuer et al. 2014; Belair et al. 2016a and 2016b; Nguyen et al. 2017; Toimela et al. 2017; Pauty et al. 2018; van Duinen et al. 2019a and 2019b; Zurlinden et al. 2020], pluripotent stem cells induced to endothelial differentiation [Belair et al. 2015; Sinha and Santoro, 2018; Li et al. 2018; Galaris et al. 2021], and endothelial-specific reporter zebrafish [Tran et al. 2007; Shirinifard et al. 2013; Tal et al. 2014 and 2017; Beedie et al. 2017; McCollum et al. 2017]. An integrated portfolio of assays is thus available to cover many aspects of the angiogenic cycle and its ramifications during neurovascular development [Bautch and James, 2009; Eichman and Thomas, 2013; Saili et al. 2017; Uwamori et al. 2017; van Duinen et al. 2019; and Zurlinden et al. 2020]. Evidence is also available to support the essentiality of this AOP outside the embryo proper, such as uterine angiogenesis [Douglas et al. 2009; Araujo et al. 2021], placentation [Abbott and Bucklew, 2000; Chen and Zheng, 2014], and human pregnancies complicated by preeclampsia and small-for-gestational age infants [Andraweera et al. 2012].

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

Term	Scientific Term	Evidence	Links
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 VEGFR system is an important molecular regulator of physiological and pathological blood vessel development. The central players are vascular endothelial growth factor receptors (VEGFR1, VEGFR2, VEGFR3) and five VEGF ligands that bind and activate these receptors during vasculogenesis, angiogenesis and lymphogenesis [Shibuya, 2013]. The MIE:305 target, VEGFR2, belongs to Class IV transmembrane receptor tyrosine kinases (RTKs) that play critical roles in the origin and progression of many adverse outcomes linked to vascular biology. Direct evidence supporting its role in developmental angiogenesis comes from functional inactivation in mouse VEGFR knockout models. For example, a targeted mutation in flt-1 showed *Vegfr1(-/-)* embryos formed endothelial cells in both embryonic and extra-embryonic regions but assembled these cells into abnormal vascular channels and died in utero at mid-somite stages [Fong et al. 1995]. Functional inactivation of *flk-1* showed that *Vegfr2(-/-)* embryos died much earlier due to deficiencies in hematopoeisis and organized blood vessels [Shalaby et al. 1995]. It's endogenous ligand, Vascular Endothelial Growth Factor-A (VEGF-A), in particular the $VEGF_{165}$ splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. Mouse embryos heterozygous for the *Vegf* gene died from impaired angiogenesis and hematopoeisis in *Vegf(+/-)* heterozygotes during organogenesis [Ferrara et al. 1996]. Nullizygotes died earlier showing that progressive severity in a quantitative gene dose-dependent manner [Carmeliet et al. 1996]. VEGF-A is a soluble protein that acts directly on endothelial cells and their precursors through VEGFR1 (*Flt-1*) and VEGFR2 (*KDR/Flk-1*). The former is a decoy receptor that traps VEGF-A into corridors preventing interaction with the active receptor, VEGFR2 [Roberts et al. 2004]. Environmental stressors (drugs/chemicals) may perturb VEGFR-dependent angiogenesis [Belair et al. 1996a,b]. Multiple mechanisms are involved, including direct effects on VEGFR2 structure-function as well as VEGF-A bioavailability or binding kinetics [Gustafsdottir et al. 2008]. The duality is relevant to MIE:305 because receptor affinity for VEGF is ten-fold higher at VEGFR1, whereas 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]. In this AOP, decreased VEGFR2 binding is the quantitative basis for an effect of stressors on VEGFR2 activation of the 'master switch' in developmental angiogenesis.

How it is Measured or Detected

A number of targeted and high-throughput assays are used to quantitively assess chemical effects leading to reduced VEGFR2 activity. Starting with VEGF availability as a preceding event, a cell-based reporter gene assay has screened approximately 73,000 compounds in a quantitative high-throughput screening (HTS) approach [Xia et al. 2009]. That assay measures cellular VEGF-secretion in an ME-180 cervical carcinoma HRE (hypoxia-response element) reporter cell line as a genetic response to hypoxia-induced *Vegf* expression. Proximity Ligation Assays (PLAs) have been used to evaluate small molecule inhibitors of VEGF-A_{165} binding to solubilized VEGFRs [Gustafsdottir et al. 2008]. PLAs are fit for the purpose of monitoring the kinetics of formation and inhibition of ligand–receptor complexes through different mechanisms of interference with VEGF-A_{165} or its cognate binding site. This allows quantitative evaluation of the potency of chemical inhibitors based on computing half-maximal inhibitory concentrations (IC50) in concentration-response curves. The inhibition of VEGF-A_{165} binding to VEGFR2 correlated well in these assays with results obtained by measuring receptor phosphorylation following exposure to molecular probes or pharmacological reagents specific to VEGF-VEGFR2 receptor capacity and kinase activity [Gustafsdottir et al. 2008]. HTS platforms have also been used to screen neary 1,000 compounds in the ToxCast/Tox21 chemical library for effects on human VEGFR2 bioactivity (https://comptox.epa.gov/dashboard/) [Kavlock et al. 2012; Judson et al. 2016; Richard et al. 2016; Thomas et al. 2018]. This biochemical (cell-free) assay is one of 331 enzymatic and receptor signaling assays under the 'NovaScreen' (ToxCast_NVS) platform [Knudsen et al. 2011; Sipes et al. 2013]. VEGFR2 enzymatic activity is measured as an electrophoretic shift in migration of a specific fluorescein-peptide substrate to the fluorescein-phosphopeptide upo 1-hour incubation with ATP. Concentration response to a test chemical is detected by a change in activity, which may be decreased or increased depending on the nature of a drug or chemical's effect on VEGFR2 catalysis or autophosphorylation, respectively with automated curve-fits [Knudsen et al. 2011; Sipes et al. 2013]. Also, in ToxCast, a multiplex assay described under the 'BioSeek' (ToxCast_BSK) platform exists for VEGFR2 bioactivity in a cell-based co-culture system [Kleinstreuer et al. 2014]. This assay measures increased or decreased levels of VEGFR2-immunoreactive protein by ELISA in primary human umbilical vein cells (HUVEC) conditioned to simulate proinflammation with histamine and IL4. Concentration response to a test chemical is curve-fitted to indicate changes in VEGFR2 receptor density. This is one of 87 endpoints covering molecular functions relevant to toxic and therapeutic pathways generated in eight cell systems for 641 environmental chemicals and 135 reference pharmaceuticals and failed drugs [Kleinstreuer et al. 2014].

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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 most closely ties into the Gene Ontology term 'Blood Vessel Morphogenesis' (GO:0048514), defined as "*The process in which the anatomical structures of blood vessels are generated and organized. The blood vessel is the vasculature carrying blood".* The molecular control of endothelial cell behaviors during blood vessel morphogenesis requires coordinated cell migration, proliferation, polarity, differentiation and cell-cell communication [Herbert and Stanier, 2011; Blanco and Gerhardt, 2013]. Among the genes linked to this process [Drake et al. 2007] are 660 genes presently curated in The Mouse Gene Ontology Browser (http://www.informatics.jax.org/vocab/gene_ontology/, last accessed November 30, 2021). Three subordinate annotations account for 593 (89.8%) of those genes: (i) vasculogenesis (96 genes, GO:0001570, defined as "*The differentiation of endothelial cells from progenitor cells during blood vessel development, and the de novo formation of blood vessels and tubes*"; (ii) *angiogenesis (545 genes, GO:0001525, defined as "Blood vessel formation when new vessels emerge from the proliferation of pre-existing blood vessels*"; and (iii) *negative regulation of blood vessel morphogenesis (110 genes, GO:0016525, defined as "Any process that stops, prevents, or reduces the frequency, rate or extent of angiogenesis*". *Vegfr2* alone mapped to both vasculogenesis and angiogenesis, consistent with its critical pro-angiogenic role. *Vegfr1* alone mapped to negative regulation of blood vessel morphogenesis consistent with its role as an endogenous angiogenesis inhibitor.

The angiogenic state of a cell can be explained as a balance between pro- and anti-angiogenic signals. During vasculogenesis, endothelial progenitor cells (angioblasts) in the prevascular mesoderm undergo a mesenchymal-to-epithelial transition to assemble into nascent endothelial tubes. This is dependent on VEGF signaling as demonstrated by the lack of nascent tubules when the prevascular mesoderm from the early mouse embryo is treated with sFlt1 or VEGF antibodies [Argraves et al. 2002] and in *vegfaa(-/-)* zebrafish embryos lacking *de novo* assembly of angioblasts into major blood vessels (dorsal aorta, cardinal vein) [Jin et al. 2019]. The acquisition of arterial or venous fate during angioblast assembly occurs during vasculogenesis [Herbert and Stanier, 2011]. While VEGFA-signaling promotes arterial fate [Jin et al. 2019], it is not required by endothelial cells to maintain their organization as an endothelium and acquire arterial or venous fates [Argraves et al. 2002]. VEGFR1 plays a role in endothelial organization and prevents overgrowth but is not required for endothelial differentiation [Fong et al. 1995; Roberts et al. 2004]. The dynamics of endothelial sprouting from existing vasculature (angiogenesis) takes over from here. VEGF signaling induces filopodial extensions to sprout from extant endothelial cells at the site, forming an endothelial tip cell (EC-tip) as the critical VEGFR2-responsive event [Belair et al. 2016a and 2016b]. Together with lateral inhibition by Dll4-Notch signaling, the VEGF-Notch-Dll4 signaling system determines where the endothelium will sprout an EC-tip cell or stay behind as a proliferating EC-stalk cells [Williams et al. 2006; Oladipupo et al. 2011; Venkatraman et al. 2016]. Angiogenic sprouts migrate along VEGF corridors established by local signals and extracellular matrix interactions, lumenize to endothelial tubules, and form connections with other tubules [Herbert and Stanier, 2011]. This requires local suppression of cell motility, pruning of any overgrowth by apoptosis, and the formation of new cell-cell junctions [Eilkin and Adams, 2010]. VEGF primes the endothelium to respond to factors that promote EC-tip cells, tubulogenesis, cytoskeletal remodeling, basement membrane deposition, activation of focal adhesion, and pericyte recruitment and proliferation [Bowers et al. 2020]. VEGF priming requires VEGFR2, and the effect of VEGFR2 is selective to the priming response. Although the genetic signals and responses for vasculogenesis (*de novo* assembly of angioblasts) and angiogenesis (endothelial growth and sprouting) differ, MIE:305 is common to both processes embedded in KE:28.

How it is Measured or Detected

Methods to quantify angiogenesis are essential to management of neovascularization for disease progression, drug discovery, and assessing environmental chemicals. Diverse assays used to detect or measure the biological states represented in KE:28 broadly stated include: (i) *in vitro* measures from endothelial cell culture, pluripotent stem cells, automated high-throughput screening (HTS) platforms, high-content imaging of human endothelial cell reporter lines, and engineered microsystems; (ii) *in vivo* measures with endothelial reporter zebrafish lines, chick chorioallantoic membrane vascularization, and genetic mouse models; and (iii) *in silico* computational models for quantitative simulation and biological integration. Each has advantages and limitations for dissecting the biological complexity of blood vessel morphogenesis, which involves coordinated control of endothelial cell migration, proliferation, polarity, differentiation, and cell-cell communication [Herbert and Stanier, 2011; Irwin et al. 2014]. *In vitro* models to study activation of endothelial function and screen for angiogenesis inhibitors are optimized to detect effects such as EC- tip cell selection, sprout formation, EC-stalk cell proliferation, and ultimately vascular stabilization by support cells [Belair et al. 2016a].

Angiogenic sprouting: Pro-angiogenic signals such as VEGF promote endothelial motility, filopodia extension and proliferation, and, together with Notch

signaling, controls whether specific endothelial cells become lead tip cells (EC-tip) or trailing stalk cells (EC-stalk) [Eilken and Adams, 2010]. During sprouting, a highly motile EC-tip cell migrates from the blood vessel and is trailed by proliferating EC-stalk cells that form the body of the nascent sprout. Chemotactic, haptotactic, and extracellular matrix (ECM) guide and support this migration; however, regulation converges ultimately on cytoskeletal remodeling in EC-tip cells that can be visualized with molecular probes and immunochemical reagents specific for actin (microfilaments) and tubulin (microtubules) [Lamalice et al. 2007]. Functional assays used to evaluate angiogenic sprouting must, however, incorporate natural (ECM) or synthetic (hydrogel) matrices to support growth factor-dependent endothelial cell proliferation, migration and VEGF-dependent invasive behaviors. Several traditional and newer methods have been used to meet that requirement.

Aortic explants: Aortic explants cultured from developing chick embryos or mouse/rat fetuses have been used as a source for evaluating drug/chemical effects on microvessel outgrowth [Baker et al. 2011; Beedie et al. 2015; Ellis-Hutchings et al. 2017; Kapoor et al. 2020; Katakia et al. 2020]. Microvascular streams from these explants are amenable to morphometric analysis of many sprouting behaviors, including cell migration, proliferation tube formation, branching, perivascular recruitment and remodeling. Sandwiching the explants in a 3D collagen matrix supplemented with optimal conditions for endothelial culture improves the spatial dimensionality of microvessel imaging [Kapoor et al. 2020]. An advantage of this platform is in its simplicity and capacity to monitor sprouting behaviors in explants sampled from different species, anatomical spaces, or stages of development [Katakia et al. 2020]. A disadvantage is that explants require animal resources in the first place.

Human cell-based *in vitro* tubulogenesis assay: Angiogenic sprouts 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. *In vitro* assays for this assembly, commonly referred to as tubulogenesis, use human umbilical vein endothelial cells (HUVEC) co-cultured with fibroblasts [Bishop et al. 1999]. Routine cell culture methods support the organization of isolated HUVEC cells into endothelial networks that resemble a microvascular bed upon stimulation with VEGF. The standardized assay detects pro-angiogenic and anti-angiogenic activities that are tracked with with immunochemical biomarkers (eg, PECAM-1) and quantified by image analysis [Bishop et al. 1999]. Refinements improved the standardized assay to increase sensitivity (limits of detection and linearity of response), reliability (reproducibility and repeatability), and predictivity for human-relevant high-throughput testing [Sarkanen et al. 2010 and 2012; Huttala et al. 2015]. The improved platform was validated in a GLP laboratory following the *OECD Guidance Document 34 for the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* [Toimela et al. 2017]. A vascular sprouting assay that utilizes mouse embryonic stem cells differentiated into vascularized embryoid bodies has been described, where the microsystem cultured onto 3D-collagen gels recapitulates key features of *in vivo* sprouting including endothelial EC-tip cell selection, migration and proliferation, polarized guidance, tubulogenesis, and mural cell recruitment [Galaris et al. 2021].

Engineered microtissues: To better recapitulate angiogenesis *in vivo*, *in vitro* assays for drug and chemical screening must adopt physiological relevant culture conditions with robustness and scalability. Human endothelial lines have been derived from induced pluripotent stem cells (iPSC-EC) and cultured in engineered platforms that mimic the 3D microenvironment [Belair et al. 2015]. They formed VEGF-dependent 3D perfusable vascular networks when co-cultured with fibroblasts and aligned with flow in microfluidic devices [Belair et al. 2015]. Encapsulating endothelial cells at controlled densities in hydrogel microspheres surrounded by a synthetic ECM [Belair et al. 2016a] or VEGF-binding peptides [Belair et al. 2016b] can be used to evaluate the activation by ECM and ECM-sequestered VEGF and other angiogenic factors. Synthetic hydrogels proved advantageous over Matrigel for consistency in screening for drug/chemical effects [Nguyen et al. 2017]. Applying an array of individually addressable microfluidic circuits to differentiating EC-tip cells in a 3D collagen enables continuous exposure to VEGF-165 and other test agents for optimizing conditions for directional sprouting, microvascular anastomosis, and vessel maturation [van Duinen et al. 2019]. The 3D micro-perfusion angiogenesis assay showed similar performance between primary endothelial cells and iPSC-ECs with regards to sprouting behaviors (eg, EC-tip cell formation, directional sprouting, and lumenization) as well as VEGF gradient-driven angiogenic sprouting [van Duinen et al. 2020]. The role of VEGF-priming has been precisely defined for serum-free 3D microvessel formation using a cocktail of growth factors needed in combination [Bowers et al. 2020]. VEGF failed to support this process under serum-free conditions but an 8-hour pretreatment with VEGF-165 led to marked increases in the endothelial cell response to angiogenic factors.

Computational models: These aspects of angiogenic sprouting have been modelled *in silico* mathematically or computationally, probing deeply into the molecular control of tip/stalk switching dynamics linked to the VEGF-Notch-DLL4 signaling [Venkataraman et al. 2016], uncovering the critical determinants of EC-tip and EC-stalk differentiation that influence the morphology of sprout progression [Palm et al. 2016], establishing canonical growth trajectories in normal and chemical-disrupted zebrafish embryos [Shirinifard et al. 2013], and simulating cell-cell interactions in a self-organizing computer model of tubulogenesis for predictive toxicology [Kleinstreuer 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 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

Endothelial networks are necessary components of normal development. Direct evidence comes from the observation of severe dysmorphogenesis and embryolethality in genetic mouse models lacking a functional VEGF signaling pathway [Fong et al. 1995; Shalaby et al. 1995; Carmeliet et al. 1996; Maltepe et al. 1997; Abbott and Buckalew, 2000; Chan et al. 2002; Coultas et al. 2005; van den Akker et al. 2007; Eberlein et al. 2021]. These alterations may follow impairment of the primitive capillary network in the early embryo and extraembryonic membranes (vasculogenesis) or its subsequent expansion and patterning of the embryonic and placental vasculature (angiogenesis). Several anti-angiogenic compounds are known to impair these stages of vascular development across multiple vertebrate species (e.g., zebrafish, frog, chick, mouse, rat) [Tran et al. 2007; Therapontos et al. 2009; Jang et al. 2009; Rutland et al. 2009; Tal et al. 2014; Vargesson, 2015; Beedie et al. 2016; Ellis-Hutchings et al. 2017; Kotini et al. 2020]. Vascular patterning is known to be sensitive event in human pregnancy as well [Husain et al. 2008; van Gelder et al. 2010; Gold et al. 2011; Vargesson and Hootnick, 2017]. Anatomically, the stabilization and has varied themes for arterial, venous, and lymphatic channels [Beedie et al. 2017; Tal et al. 2017]. These events are mediated by angiogenic factors through receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and glycosyl phosphatidyl-inositol (GPI)-anchored receptors, and later vascular flow-mediated signals [Drake et al. 2007; Knudsen and Kleinstreuer, 2011]. These provide assayable targets for high-throughput screening (HTS) assays, and an open source of data screening hundreds of chemicals for impairment to the angiogenic cycle [Tran et al. 2007; Houck et al. 2009; Kleinstreuer et al. 2011; Knudsen et al. 2011 and 2013; Kleinstreuer et al. 2014; Tal et al. 2014 and 2017; McCollum et al. 2017; Saili et al. 2019; Zurlinden et al. 2020].

Key Event Description

In embryological terms, the angiogenic cycle entails a stepwise progression of formation, maturation, and stabilization of the microvasculature [Hanahan, 1997; Drake et al. 2007; Chung and Ferrara 2011; Knudsen and Kleinstreuer, 2011; Coultas et al. 2005; Huang, 2020]. This level of impairment of blood vessel morphogenesis best maps to Gene Ontology (GO) annotations: GO:001885 for 'endothelial cell development', which is defined as "*The progression of an endothelial cell over time, from its formation to the mature structure*"; and/or GO:0045601 for 'regulation of endothelial cell differentiation', defined as "*Any process that stops, prevents, or reduces the frequency, rate or extent of endothelial cell differentiation*". The numbers of curated genes associated with these categories in the MGI database (http://www.informatics.jax.org/vocab/gene_ontology/) are 75 genes and 44 genes, respectively, for a total of 97 genes altogether. In addition, pericyte-endothelial interactions are indispensable for maturation and stabilization via broader signaling pathways (eg, VEGFA, PDGFB, Notch-DLL4, AGPNT, Norrin, TGF-β) that have been characterized during patterning neovascularization [Azam et al. 2018; Huang, 2020]. Neovascular stabilization is an active process that requires specific cellular signaling, including pro-angiogenic pathways such as VEGF and FGF, angiopoietin-Tie2 for endothelial cell survival and junction stabilization, PDGF and TGF-β signaling that modify mural cell (pericytes, vascular smooth muscle cells) functions to fortify vessel integrity [Murakami, 2012]. Breakdown of these signaling systems results in pathological hyperpermeability and/or genetic vascular abnormalities such as vascular malformations, ultimately progressing to hemorrhage and edema. Vascular mural cells are recruited to the endothelial network by endothelial cell signals [Sinha and Santoro, 2018]. A number of anti-angiogenic compounds, including Vatalanib and Thalidomide, have been shown to impair neovascularization during developmental angiogenesis [Tran et al. 2007; Therapontos et al. 2009; Jang et al. 2009; Rutland et al. 2009; Tal et al. 2014; Vargesson, 2015; Beedie et al. 2016; Ellis-Hutchings et al. 2017; Kotini et al. 2020]. In exposed zebrafish embryos, early effects of potential vascular disrupting chemicals (pVDCs) invoke changes to the anatomical development of intersegmental vessels from the dorsal aorta [Tran et al. 2007; Tal et al. 2014; McCollum et al. 2017]. Thalidomide, for example, has been shown to primarily disrupt immature vascular networks versus more mature vasculature in the embryo [Therapontos et al. 2009; Beedie et al. 2016a, 2016b, 2017].

Evidence for KE:110 in human studies is indirect, based on the association of malformations with altered vascular patterns and exposure to anti-angiogenic drugs in women of reproductive potential or during pregnancy [Husain et al. 2008; van Gelder et al. 2010; Gold et al. 2011; Ligi et al. 2014; Vargesson and Hootnick, 2017]. Key nodes in the ontogenetic regulation of angiogenesis have been investigated with human cell-based high-throughput assay (HTS) platforms in ToxCast to screen for pVDCs acting on the formation, maturation and/or stabilization of endothelial networks [Houck et al. 2009; Knudsen et al. 2011; Kleinstreuer et al. 2014; Saili et al. 2019; Zurlinden et al. 2020].

How it is Measured or Detected

Microvascular structure: Endothelial network formation can be monitored quantitatively *in vitro* using different human cell-based angiogenesis assays that score endothelial cell migration, 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. 2017; Toimela et al. 2017; Saili et al. 2019; Zurlinden et al. 2020]. Cell types commonly employed are human umbilical endothelial cells (HUVECs) and more recently endothelial cells derived from human induced pluripotent stem cells (iPSC-ECs) through various differentiation and purification protocols [Belair et al. 2015 and 2016; Iwata et al. 2017; Bezenah et al. 2018; van Duinen et al. 2019 and 2020]. Synthetic hydrogels are shown to promote robust *in vitro* network formation by HUVEC or iPSC-ECs in response to angiogenic factors as superior sensitivity and reproducibility to detect pVDCs [Nguyen et al. 2017]. Although endothelial cell models of migration, proliferation, apoptosis, and tube formation are popular due to their simplicity and throughput, these assays lack the biological complexity of an *in vivo* system. Animal models, including the chick chorioallantoic membrane assay, corneal neovascularization assay, and 3D embedded matrices preserve biological complexity but are costly and low throughput [Tran et al. 2007]. Endothelial-specific transgenic zebrafish reporter embryos thus provide a test system that combines the biological complexity of *in vivo* models with automated high-throughput screening (HTS).

Maturation and stabilization: Chemical effects may be detected by HTS assays for phenotypic profiling in endothelial co-culture systems based on specific biomarker protein readouts [Kleinstreuer et al. 2014]. The ToxCast portfolio includes eight human cell-based systems for screening chemicals that disrupt physiologically important cell-cell signaling pathways, including vascular biology. The endpoints measured can be closely linked to *in vivo* outcomes. Local signals may act through several receptor modalities, including receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and glycosyl phosphatidyl-inositol (GPI)-anchored receptors as part of a ToxCast *in vitro* signature for profiling potential vascular disrupting compounds (pVDCs) [Knudsen and Kleinstreuer, 2011; Kleinstreuer et al. 2013; Tal et al. 2017; Saili et al. 2019].

Assessing weight of evidence with a ToxCast pVDC predictive signature assays for KE:110:

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

ToxCast HTS predictions for 38 potential pVDCs and non-pVDCs were tested across ten *in vitro* platforms from laboratories addressing different aspects of the vasculogenic/angiogenic cycle. Three tubulogenesis platforms used traditional HUVECs [Sarkanen et al. 2010; Toimela et al. 2017]; 3D endothelial sprouting and network assays used endothelial cells derived from human induced pluripotent stem cells (iPSCs) [Belair et al. 2016b; Nguyen et al. 2017; Zurlinden et al. 2020]; microvessel outgrowth in rat fetal aortic explants [Ellis-Hutchings et al. 2017] and transgenic endothelial reporter zebrafish lines [Tal et al. 2017; McCollum et al. 2017] rounded out the panel. While no single study confirmed all of the pVDC predictions, the combined vascular disrupting effects across all studies aligned well with the *in silico* predictions (87% accuracy; positive predictive value of 93%, negative predictive value of 73%) [Saili et al. 2019]. ToxCast assay features input to the prediction model were detected as follows.

Vascular cell adhesion molecule 1 (VCAM1): the pVDC signature aggregates assays from the BioMAP Systems Predictive Toxicology panel [Kunkel et al., 2004; Houck et al., 2009] 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 [Knudsen et al. 2011; Kleinstreuer et al. 2013; Tal et al. 2017; Saili et al. 2019; Zurlinden et al. 2020].

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 outgrowth: 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.

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Event: 298: Insufficiency, Vascular

Short Name: Insufficiency, Vascular

Key Event Component

Process	Object	Action

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

Embryonic blood vessels form in a reproducible pattern that interfaces with other embryonic structures and tissues [Hogan et al. 2004]. Many human diseases, including stroke, retinopathy, and cancer, are associated with the vascular biology, including endothelial cells and pericytes that establish the blood-brain barrier and control cerebrovascular exchanges [Bautch and James, 2009; Eichmann and Thomas, 2013; Saili et al. 2017]. Functionally, blood vessel morphogenesis is critical for providing oxygen, nutrients and molecular signals to developing tissues [Maltepe et al. 1997; Vargesson, 2003; Chung and Ferrara, 2011; Eshkar-Oren et al. 2015]. The developing vascular network is shaped into a hierarchical system of arteries and veins, through progressive effects on blood vessel arborization (microvasculature) and pruning (angio-adaptation) [Jin et al. 2017]. The former is morpho-regulatory whereas the reshaping is influenced by regional changes in blood flow and local metabolic demands [Tran et al. 2007]. Evidence supports the ability of physiological parameters such as oxygen and glucose concentrations to affect the expression of genes critical for developmental angiogenesis [Maltepe and Simon, 1998]. Growth in tissue mass during organogenesis is thought to lead to the formation of hypoxic/nutrient-deprived cells. The subsequent activation of sensors such as HIF-1 [Xia et al. 2009; Oladipupo et al. 2011; Li et al. 2018] and ARNT [Maltepe et al. 1997; Abbott and Buckalew, 2000] that rapidly trans-activate the expression of genes such as VEGF that drive angiogenesis.

While mammalian embryos become sensitive to hypoxia during early organogenesis, the small size of zebrafish embryos renders this species less vulnerable to hypoxia than vertebrate counterparts; however, the genetic control of microvascular development is conserved among vertebrate species as evidenced by hypoxia-responsive signaling (HIF-1) via local oxygen-sensing gradients in the zebrafish, chick and mouse embryo [Hogan et al. 2004; Liu et al. 2017; Gerri et al. 2017]. The neural tube, for example, provides vascular patterning signals that direct formation of the perineural vascular plexus (PNVP) that encompasses the neural tube at mid-gestation [Hogan et al. 2004]. This process is temporally and spatially associated with *Vegfa* expression as the neural tube signal through VEGFR-2. Mesodermal VEGFR-2 expression is localized to the lateral portion of the somite and later to sclerotomal cells surrounding the neural tube under the positive control of BMP4 signaling and negative control by Noggin, a BMP4 antagonist [Nimmagadda et al. 2005]. Reciprocal signaling between VEGF-induced endothelial cells and neuroprogenitor cells enhanced formation of the brain neurovascular unit [Vissapragada et al. 2014]. In transgenic zebrafish embryos, the VEGFR-2 antagonist, Vatalanib produced a direct concentration-dependent progression of impaired intersegmental vessel (ISV) outgrowth in early embryos, increased rates of malformed hatched larva, and reduced survival in juvenile cohorts [Tal et al. 2014]. These data show that disruption in the early embryo has a lasting impact on advanced life stages.

Another key cell sensing activity is the recruitment of macrophage (microglia?) cells that secrete pro-angiogenic cytokines and proteases, remodeling the extracellular matrix (ECM) and providing survival and guidance cues to endothelial cells [Gerri et al. 2017]. Macrophages play crucial roles at each step of the angiogenic cycle, from sprouting to maturation and remodelling of the vascular plexus through angiopoietin-TIE2 signaling [Du Cheyne et al. 2020], which is known to synergize with the VEGF-pathway during developmental angiogenesis [Li et al. 2014]. A seminal study showed that loss of immature blood vessels is the primary cause of Thalidomide-induced teratogenesis in the chick embryo, where anti-angiogenic but not anti-inflammatory analogues of Thalidomide induced limb reduction defects. Outgrowth and remodeling of more mature blood vessels delayed, whereas newly formed angiogenic vessels were lost prior to limb dysmorphogenesis and altered patterns of gene expression [Therapontos et al. 2009; Vargesson, 2015]. Vascular insufficiency is likely important in human embryos where the window of vulnerability to Thalidomide-induced phocomelia precedes full establishment of the adult arterial pattern by the 8th week of gestation [Hootnick et al. 2016; Hootnick et al. 2017; Vargesson and Hootnick, 2017].

As such, a chemical's potential to disrupt vascular patterning and/or remodeling during organogenesis can have profound effects on many systems, including: early limb development [Beedie et al. 2016a, 2016b, 2017 and 2020]; neurovascular development [Hogan et al. 2004; Hallene et al. 2006; Bautch and James, 2009; Eichman and Thomas, 2013; Vissapragada et al. 2014; Fiorentino et al. 2016; Uwamori et al. 2017; Huang, 2020]; and utero-placental development [Abbott and Buckalew, 2000; Douglas et al. 2009; Rutland et al. 2009; Chen, 2014; Araujo et al. 2021].

How it is Measured or Detected

Complex functional assays such as the rat aortic explant assay, rat whole embryo culture, and the zebrafish embryotoxicity 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].

Zebrafish reporter assays: Blood flow begins in the zebrafish embryo at ~24 h postfertilization. Shortly after this, the angiogenic vessels that perfuse the trunk of the embryo (intersegmental vessels) sprout from the vasculogenic vessels [Tran et al. 2007]. These effects can be visualized in automated, quantitative screening assays using transgenic zebrafish expressing green fluorescent protein (GFP) under the control of the vascular endothelial growth factor receptor (VEGFR) *Vegfr2* promoter that restricts reporter gene expression to developing blood vessels. Phenotypic readouts have 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]. 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 relative to controls during chemical exposure [Tal et al. 2017].

ToxCast: A study evaluated two anti-angiogenic agents, 5HPP-33, a synthetic Thalidomide analog [Noguchi et al. 2005] and TNP-470, a synthetic Fumagillan analog [Ingber et al. 1990] across the ToxCast HTS assay platform and anchored the results to complex *in vitro* functional assays: the rat aortic explant assay, rat whole embryo culture, and zebrafish embryotoxicity [Saili et al. 2019]. Both compounds disrupted angiogenesis and embryogenesis in the functional assays, with differences in potency and adverse effects. 5HPP-33 was embryolethal, whereas TNP-470 produced caudal defects at low concentrations [Ellis-Hutchings et al. 2017]. Anti-angiogenic modes of action are known for 5HPP-33, which blocks tubulin polymerization inhibition [Yeh et al. 2000; Inatsuki et al. 2005; Kizaki et al. 2008; Rashid et al. 2015); and TNP-470, a methionine aminopeptidase II (MetAP2) inhibition, through non-canonical Wnt inhibition of endothelial proliferation [Ingber et al. 1990]. Transcriptomic profiles of exposed embryos pathways unique to each and in common to both, strongest being the TP53 pathway [Saili et al. 2019]. In mouse, TNP-470 reduced fetal intraocular microvasculature and induced microphthalmia, either directly or via effects on placental morphology [Rutland et al. 2009].

Computational models: Critical pathways for developmental angiogenesis and potential disruptions have critical signal-response systems embedded in three types of receptors that play key roles in a number of morphoregulatory processes: receptor tyrosine kinases (e.g., growth factor-signaling), G-protein coupled receptors (e.g., chemokine signaling), and GPI-anchored receptors (e.g., uPAR system). Computational approaches have been used to predict vascular insufficiency for potential vascular disrupting chemicals (pVDCs) that are developmental toxicants or non-toxicants [Kleinstreuer et al. 2011; Knudsen and Kleinstreuer, 2011]. This has been applied to the ToxCast inventory to rank order over a thousand chemicals for validation testing [McCollum et al. 2017; Tal et al. 2017; Saili et al. 2019; Zurlinden et al. 2020].

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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

Domain of Applicability: Maternal and fetal weight effects and viability were the most often affected parameters at the developmental lowest effect levels, followed by skeletal malformations [Knudsen et al. 2009; Rorije et al. 2012]. Specific endpoints such as phocomelia have critical value in setting regulatory decisions for drugs and chemicals; however, they are less frequently observed than fetal weight reduction or skeletal malformations. 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). Viability after delivery is important outcome for human health concerns, as are other conditions that may be missed in OECD 414 (e.g., stillbirth and neonatal mortality, long-term neurologic handicap, and maternal mortality). Those relevant to AO:1001 may be captured in the one-or two-generation reproduction toxicity study designs (OECD 415 and 416, respectively).

Key Event Description

Key Event Description: The risks for chemical effects on the reproductive cycle in mammals are broadly defined in two categories for regulatory purposes: reproductive (fertility, parturition, lactation) and developmental (mortality, malformations, growth and functional deficits). Many advances in our knowledge of fundamental human embryology derives from model organisms such as zebrafish and chick embryos [Beedie et al. 2016 and 2017]. The standard formulation of prenatal developmental toxicity for drug or chemical exposure underscores several dependencies: initiating mechanisms (targets); dose response (quantitative response); stage susceptibility (temporal response); species differences (concordance); chemical bioavailability (metabolism and kinetics); and apical endpoint (phenotype). These principles have continued to guide scientific research in teratology, are widely used in teaching [Friedman, 2010].

How it is Measured or Detected

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 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.

Regulatory Significance of the AO

Regulatory Significance of the Adverse Outcome: 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 two-species testing paradigm was developed in response to the pandemic of phocomelia associated with maternal exposure to thalidomide during early pregnancy [Schardein 2000]. 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 specific manifestations of embryo-fetal toxicity may in general vary greatly between species, and even between strains within the same species [Hurtt et al. 2003; Janer et al. 2008; Theunissen et al. 2016].

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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

Domain of Applicability: The *de novo* assembly of endothelial cells into the primitive capillary network in an early embryo (vasculogenesis) or a tubular network in vitro (tubulogenesis) are both driven by VEGF-A signaling. A critical effect on developmental angiogenesis aligns with the Gene Ontology (GO) term 'negative regulation of blood vessel morphogenesis' (GO:0016525), defined as "*Any process that stops, prevents, or reduces the frequency, rate or extent of angiogenesis*". Differences exist among the 110 genes mapped to this annotation in the Mouse Gene Ontology Browser (http://www.informatics.jax.org/vocab/gene_ontology/, last accessed November 30, 2021). Although the genetic signals and responses may differ between vasculogenesis and angiogenesis [Drake et al. 2007; Knudsen and Kleinstreuer, 2011], disruption of the former process ultimately leads to a reduction in the latter during development and so both are in the DoA for this KER.

Key Event Relationship Description

VEGF signals promote endothelial cell motility, filopodial extension and proliferation, and together with Notch signaling controls whether specific endothelial cells (ECs) become pioneering 'EC-tip' cells (non-proliferating) or trailing 'EC-stalk' cells (proliferating). VEGFR2 activation is the master switch that promotes motility and exploratory behaviors of leading EC-tip cells and a mitogenic effect on trailing EC-stalk cells [Ellken and Adams, 2010; Herbert and Stanier 2011; Blanco and Gerhardt, 2013]. An early step is EC-tip cell selection [Eilken and Adams, 2010]. Endothelial cells are normally suppressed in their tip cell behaviors by Notch-Delta signaling [Blanco and Gerhardt, 2013; Li et al. 2014]. This lateral inhibition is broken when VEGFR2 is activated by VEGF-A. Delta-like 4 (Dll4), a membrane-bound ligand for Notch1 and Notch4, is selectively expressed in response to VEGF-A induction. This down-regulates VEGFR-2 expression in prospective EC-stalk cells but promotes VEGFR2 expression in EC-tip cells, enabling them to extend filopodial processes along VEGF-A rich paths thus orienting the angiogenic sprout [Williams et al. 2006]. VEGF-A rich corridors are established during *in vivo* development by local VEGFA gradients and the distribution of soluble VEGFR-1, a so-called 'decoy receptor' sequestered and released during enzymatic remodeling of ECM, both serving to channel sprouting progression along VEGFA-rich corridors [Roberts et al. 2004; Chappell et al. 2009 and 2016].

Evidence Supporting this KER

Biological Plausibility: The control of EC-tip cell dynamics is a central feature linking VEGFR-2 inhibition (MIE:305) to adverse angiogenic sprouting behaviors (AE:28) [Argraves et al. 2002; Williams et al. 2006; Eilken and Adams, 2010; Oladipupo et al. 2011; Venkatraman et al. 2016; Beloglazova et al. 2021].

Empirical Evidence: Vascular endothelial growth factor-A (VEGF-A), in particular the $VEGF_{165}$ splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. This is evidenced in time-scale relationships for immature blood vessel formation and embryonic lethality in mutant mouse embryos heterozygous for the *Vegfa*-null allele [Ferrara et al. 1996; Carmeliet et al. 1996]. Targeted disruption of genes encoding VEGFR1 or VEGFR2 are also 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]. The requirement of VEGFA signaling is relevant to KER:335 for angiogenesis not only during embryonic development but for the uterine cycle, pregnancy, wound healing, and tumorigenic vessel growth in the adult. The inferred 'window of vulnerability' for chemical teratogenesis involves key events during early postimplantation stages of human development.

Uncertainties and Inconsistencies: Many physiological states 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 other signaling system [Knudsen et al. 2016].

Crosstalk between VEGFR-2 and other pro-angiogenic receptor tyrosine kinase (RTK) activities such as PDGFR or FGFR is known. This crosstalk has been embraced in the search for clinically efficacious synergistic kinase anti-angiogenesis strategies in suppressing tumorigenic growth [Lin et al. 2018] but is an uncertainty for establishing a role for KER:335 in the disruption of blood vessel morphogenesis (KE:28). For example, the fungal metabolite Epoxyquinol B inhibits kinase activity across several RTKs including VEGFR and PDGFR and blocks VEGF-induced migration and tubulogenesis in human umbilical vein endothelial cells (HUVECs) [Kamiyama et al. 2008]. Anlotinib inhibits cell migration and microvessel formation in the rat aortic ring assay and chicken chorioallantoic membrane assay via the ERK signaling pathway in both species [Lin et al. 2018]. Derazantinib at 0.1 μM to 3 μM blocked intersegmental vessel (ISV) migration linked to VEGF, PDGF, or FGF pathways in zebrafish embryos [Kotini et al. 2020].

Still other pathways may be relevant with regards to developmental angiogenesis. For example, the endothelial TIE2 receptor is essential for ISV outgrowth in zebrafish embryos [Li et al. 2014] and TGFβ1 signaling in the formation of tubular networks in human vascular endothelial cells (HUVECs) [Zhang et al. 2021]. VEGF-dependent cell migration in HUVECs is also facilitated by the urokinase-type plasminogen activator receptor (uPAR), a system linked to cell-ECM interactions and Notch components: Notch1 receptor and ligands (Dll1, Dll4, Jag1) in endothelial cells on one hand, and uPA, uPAR, TGFβ1, integrin β3, Jag1, Notch3 receptor in mural cells on the other hand [Beloglazova et al. 2021]. Both an increase on pro-angiogenic factors as well as a decrease in anti-angiogenic factors (Notch signaling) can have similar outcomes. Crosstalk in these heterogeneous systems point to cell-specific patterns of gene expression as a critical determinant of RTK expression and cell-type specificity. As such, quantitative linkages to VEGF signaling must consider the uncertainties from effects to other MIEs.

Quantitative Understanding of the Linkage: Studies with pharmacological VEGFR2 inhibitors have shown their concentration dependent effect on angiogenic sprouting. For example, the VEGFR2 antagonist Vatalanib (PTK787) suppressed zebrafish ISV outgrowth in a concentration-dependent manner that was characterized quantitatively at 72 hours post-fertilization (hpf) and became evident at the 0.07 μM concentration level [Tal et al. 2014]. An even lower concentration of Vatalanib (0.01 μM) inhibited angiogenic sprouting dynamics in a 3D microsystem of human endothelial cells derived from induced pluripotent stem cells (iPSC-ECs) [Belair et al. 2016b]. The response-response relationship for Vatalnib in zebrafish was maintained for dysmorphogenesis at 120 hpf (0.22 μM) and adult survival curves at 10 days (0.70 μM) [Tal et al. 2014]. While Vatalanib inhibits both VEGFR-2 and PDGFRβ, it is most selective for VEGFR-2 [Wood et al. 2000].

Shirinifard et al. [2013] examined angiogenic sprouting dynamics in zebrafish embryos exposed to high concentrations of arsenic (As). This resulted in a suppressed but chaotic pattern of ISV outgrowth. Quantitative mathematical models inferred increased exploratory filopodial behaviors of EC-tip cells accounting for the loss of directional sensing of during ISV outgrowth [Shirinifard et al. 2013]. The chaotic versus ordered EC-tip cell dynamics may be mechanistically linked to key modulatory factors that regulate the cytoskeletal cycle and/or cell-ECM biomechanics. Molecular pathways such as the Aryl hydrocarbon receptor (AhR) and hypoxia-inducible factor-1 alpha (HIF-1α) that control genes in response to xenobiotic metabolism, hypoxia, and hypoglycemia have potential feedback roles. These pathways regulate genes in developmental angiogenesis. For example, functional inactivation of ARNT, the AhR nuclear translocator protein, results in critical embryonic vascular phenotypes in the yolk sac and branchial arches reminiscent of those observed in mouse embryos deficient in VEGF-signaling [Maltepe et al. 1997].

Domain of Applicability: The *de novo* assembly of endothelial cells into the primitive capillary network in an early embryo (vasculogenesis) or a tubular network in vitro (tubulogenesis) are both driven by VEGF-A signaling. A critical effect on developmental angiogenesis aligns with the Gene Ontology (GO) term 'negative regulation of blood vessel morphogenesis' (GO:0016525), defined as "*Any process that stops, prevents, or reduces the frequency, rate or extent of angiogenesis"*. Differences exist among the 110 genes mapped to this annotation in the Mouse Gene Ontology Browser (http://www.informatics.jax.org/vocab/gene_ontology/, last accessed November 30, 2021). Although the genetic signals and responses may differ between vasculogenesis and angiogenesis [Drake et al. 2007; Knudsen and Kleinstreuer, 2011], disruption of the former process ultimately leads to a reduction in the latter during development and so both are in the DoA for this KER.

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linked to cell-ECM interactions and Notch components: Notch1 receptor and ligands (Dll1, Dll4, Jag1) in endothelial cells on one hand, and uPA, uPAR, TGFβ1, integrin β3, Jag1, Notch3 receptor in mural cells on the other hand [Beloglazova et al. 2021]. Both an increase on pro-angiogenic factors as well as a decrease in anti-angiogenic factors (Notch signaling) can have similar outcomes. Crosstalk in these heterogeneous systems point to cell-specific patterns of gene expression as a critical determinant of RTK expression and cell-type specificity. 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

Blood vessel morphogenesis requires coordinated control of endothelial cell (EC) and supportive mural cells staged to develop interconnected networks required for a fully functional circulatory system. Formation of endothelial networks *in vivo* and *in vitro* are dependent on VEGF-Notch-Dll4 signaling that determines EC specification and sprouting outgrowth to form microvessels that lumenize for blood circulation. Cell motility, proliferation, differential cell adhesion) are indispensable for multicellular tubular networks to emerge *in vivo* or *in vitro* [Nguyen et al. 2017; Toimela et al. 2017; Pauty et al. 2018; van Duinen et al. 2019a and 2019b; Zurlinden et al. 2020]. In HUVEC cells, VEGFR2 activates phospholipase PLCβ3 generating a second messenger (inositol-3-phosphate) that promotes EC migration (CDC42 activation) and suppresses EC proliferation (cell cycle progression) [Bhattacharya et al. 2009]. The ephrins couple VEGF signaling to endothelial patterning [Patan, 2000]. Unlike VEGFR2 activation, EPH-class receptor tyrosine kinase activation requires direct contact between cells expressing a receptor (EPH) and complementary ligand (EFN). Ephrin-B4 expression (*Efnb4*) in the mouse embryo co-localizes with its *Ephb2* receptor in developing arterial endothelial cells and with its *Ephb4* receptor in prospective venous endothelial cells. This partitioning of prospective arterial and venous counterparts stimulates microvascular density [Wang et al. 1998]. A ToxCast signature for embryonic vascular disruption (pVDCs) built with bioactivity profiling data from functional assays on genes for developmental angiogenesis was 87% accurate when anchored to empirical observations on 38 chemicals summed across 10 *in vitro* platforms across endothelial network formation [Saili et al. 2019].

Evidence Supporting this KER

Biological Plausibility: Endothelial network formation is dependent on proper regulation of angiogenic sprouting. Cell migration requires precise control, which is altered or lost when tumor cells become invasive and metastatic [Muller et al. 2002].

Empirical Evidence: Compounds that disrupt angiogenic sprouting behaviors [Belair et al. 2016] also disrupt endothelial tubular network formation [Nguyen et al. 2016]. Activation of VEGFA signaling expands the arterial cell population at the expense of venous cells during vasculogenesis of the axial vessels in zebrafish; *Vegfa* deficiency interferes with the pathfinding of intersegmental vessels (ISVs) and a loss of a cranial vasculature [Jin et al. 2017]. A zebrafish embryo vascular model in conjunction with a mouse endothelial cell model revealed a plethora of vascular perturbations including malformed ISVs, uncondensed caudal vein plexus, hemorrhages and cardiac edema [McCollum et al. 2017]. Ephrin-B4 expression (*Efnb4*) in the mouse embryo co-

localizes with its *Ephb2* receptor in developing arterial endothelial cells and with its *Ephb4* receptor in prospective venous endothelial cells. This partitioning of prospective arterial and venous counterparts stimulates microvascular density [Wang et al. 1998].

Uncertainties and Inconsistencies: Downregulating the VEGF signaling pathway in early zebrafish embryos, while affecting the number of angioblasts, did not appear to affect their migratory behaviors [Jin et al. 2005]. These findings indicate that chemical effects on developmental angiogenesis may be cell-specific, stage-dependent, and regionally selective. The progression of chemical effects on blood vessel morphogenesis *in vivo* is complicated by uncertainties that reflect the recovery potential or natural selection of an exposed embryo. Improved molecular understanding is necessary to understand the complex variables for these effects.

Quantitative Understanding of the Linkage: A ToxCast signature for potential Vascular Disrupting Chemical (pVDC) [Knudsen and Kleinstreuer, 2011; Kleinstreuer et al. 2013] has been tested for predictivity [Saili et al. 2019]. The pVDC signature included biochemical features for three receptor systems prominent in developmental angiogenesis (receptor tyrosine kinases for growth factor signals; the urokinase-type plasminogen activator (uPA) system that functions in VEGFR2-induced changes to focal adhesion and extracellular matrix (ECM) degradation during sprout progression; and G-protein coupled receptors (GPRCs) for angiogenic cytokines and chemokines) [Knudsen et al. 2011; Sipes et al. 2013; Kleinstreuer et al. 2014] (see image below). The battery of assays represented 21 ToxPi slices (see below) for a ToxPi [Marvel et al. 2018] based profile of Aop43 in sectors for G-protein coupled receptors (red-orange), receptor tyrosine kinases (blue-purple), and uPAR system (green-yellow) [Knudsen and Kleinstreuer, 2011; Kleinstreuer et al. 2013]. 38 ToxCast chemicals were selected for targeted testing by different laboratories having expert-qualified in vitro assays that are sensitive to, or specific for, different stages of the angiogenesis cycle (e.g., activation, sprouting, migration, tubulogenesis, vascular patterns). The ToxPi prediction was 87% accurate when in vitro observations were summed across all 10 platforms [Saili et al. 2019]. This shows the value of Aop43 in combining HTS data from ToxCast with biological knowledge of the angiogenesis cycle derived from curated knowledge from genetic mouse models – in this case for developmental angiogenesis, that establishes a course of predictivity from sprouting to patterning [Saili et al. 2019]. The U.S. EPA SeqAPASS tool revealed how the genetic signature may have evolved phylogenetically [Tal et al. 2017].

Response-response Relationship: Consequences of Vatalnib exposure to early zebrafish embryos was maintained for inhibition of ISV sprouting progression (0.07 µM) at 72 hours post-fertilization (hpf), dysmorphogenesis at 120 hpf (0.22 µM), and adult survival at 10 days (0.70 µM) [Tal et al. 2014]. The progression of critical concentrations through development and adult stages may be explained by recovery or natural selection processes.

Known modulating factors: The importance of canonical and non-canonical Wnt signaling in embryonic development and tissue homeostasis is widely known for its ability to influence cell movement, ECM degradation and paracrine signaling [Sedgwick et al. 2016]. Differences in Wnt signaling could, for example, contribute to the differential recovery processes in the embryo across space and time.

Domain of Applicability: Morphology of endothelial networks with regards to their completeness and complexity is a feature dependent on cell-cell signaling within the endothelial network as well as their microenvironment with regards to the ECM and other cell types. A critical effect on developmental angiogenesis aligns with the Gene Ontology (GO) term GO:001885 'endothelial cell development', which is defined as "*The progression of an endothelial cell over time, from its formation to the mature structure*" and/or GO:0045601, 'regulation of endothelial cell differentiation', defined as "*Any process that stops, prevents, or reduces the frequency, rate or extent of endothelial cell differentiation*". Differences exist among the 119 genes mapped to this annotation in the Mouse Gene Ontology Browser (http://www.informatics.jax.org/vocab/gene_ontology/, last accessed November 30, 2021).

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

Evidence Supporting Applicability of this Relationship

Mammalian Phenotype Browser (MPO) defines 'abnormal blood vessel morphology' (MP:0001614) as "*any structural anomaly of the network of tubes that carries blood through the body*". They describe abnormalities linked to: (i) specific cell types of the microvasculature (endothelial cells, pericytes, macrophages); (ii) diversification of arterial, venous, and lymphatic channels; and (iii) organ-specific vascular morphologies including malformations, variations, and pathologies. The subordinate term 'abnormal vascular development' (MP:0000259) defines an "*aberrant process of vascular formation*" that neatly captures the biology relevant to this KER. There are 1045 genotypes and 1768 annotations associated with this term (last accessed December 24, 2021).

Key Event Relationship Description

An embryo develops normally only with an adequate supply of oxygen, nutrients, molecular signals, and removal of waste products [Maltepe et al. 1997]. In its early stages this may be satisfied by simple diffusion; however, the rate of diffusion becomes limiting beyond a certain mass. The circulatory system becomes functional early in development and is the first organ system to operate in the vertebrate embryo, reflecting this critical role during organogenesis [Chan et al. 2002; Jin et al. 2005; Walls et al. 2008]. With the onset of cardiac function during early organogenesis the primitive vascular system quickly evolves into a patent circulatory system that transports hematopoietic cells through major blood vessels (e.g., dorsal aorta, cardinal veins, and six aortic arches in the branchial region). Impaired endothelial formation impacts this role in many ways through abnormalities in artery/vein development, vascular remodeling, tissue neovascularization, and microvascular ramifications.

Evidence Supporting this KER

Biological Plausibility

Biological Plausibility: Problems of insufficient blood support due to slow or weak heartbeat, vessel occlusions, or anemia will take a toll on various organ systems depending on the stage of development and regional responses to oxygen-sensing pathways [Maltepe et al. 1998; Liu et al. 2009; Gerri et al. 2017].

Empirical Evidence

Empirical Evidence: Microvascular specializations derived from the perineural vascular plexus (PNVP) surrounding the neural tube and choriovitelline system (CVS) in extraembryonic membranes establish critical transport interfaces with the CNS (e.g., blood-brain barrier and retinal vascularization) [Dorrell et al. 2002; Hogan et al. 2004; Bautch and James, 2009; Eichmann and Thomas, 2013; Vissapragada et al. 2014; Fiorentino et al. 2016; Uwamori et al. 2017; Saili et al. 2017; Huang, 2020] and extraembryonic environment [Abbott and Buckalew, 2000; Chen and Zheng, 2014], respectively. These systems are particularly vulnerable to problems of unstable and leaky vessels in otherwise well-defined endothelial networks.

Uncertainties and Inconsistencies

Uncertainties and Inconsistencies: Blood flow patterns vary in higher vertebrates as vascular anatomy becomes complicated by asymmetrical loss of some vessels and expansion of others, especially in mammals where prenatal circulatory shunts bypass the fetal lungs and liver due to placental function.

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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 Applicability of this Relationship

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 individual 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 Relationship Description

Blood vessels in a developing embryo change to accommodate rapid growth, morphogenesis and differentiation. The importance of development and maintenance of the vasculature is evident in the association between developmental defects and vascular insufficiency, particularly arterial dysgenesis, derived by experimental teratogenesis and inferred in clinical teratology [Vargesson and Hootnick, 2017]. Several known anti-angiogenic compounds have been shown to cause dose-dependent developmental defects in various animal models (e.g., zebrafish, frog, chick, mouse, rat) [Therapontos et al. 2009; Jang et al. 2009; Rutland et al. 2009; Tal et al. 2014; Vargesson, 2015; Beedie et al. 2016; Ellis-Hutchings et al. 2017; Kotini et al. 2020]. Human studies of malformations showed a correlation with genetic and/or environmental factors that target vascular development [Husain et al. 2008; Gold et al. 2011]. Broad analysis of medicinal compounds to which women of reproductive age were exposed identified ‘vascular disruption’ as one of six potential mechanisms of teratogenesis [van Gelder et al. 2010].

Evidence Supporting this KER

Biological Plausibility: A failure of correct vessel patterning, vessel occlusion in the embryo, or placental defects limiting maternal-fetal nutrition could result in tissue damage to an embryo invoking malformations and other developmental defects at critical periods of development. This perhaps best known for limb reduction defects (e.g., phocomelia) following thalidomide exposure during early limb development, when the critical response coincides with nascent vascular patterning prior to innervation [Therapontos et al. 2009]. At this stage, the early limb-bud receives its blood supply from a single axial artery at which time the undifferentiated mesenchyme is perfused by a simple capillary network. Susceptibility to thalidomide-induced dysmorphogenesis declines as the vascular pattern transitions to a more complex and definitive system of maturing vessels and emergence of the skeletal elements [Vargesson and Hootnick, 2017].

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Empirical Evidence

Empirical Evidence: Two lines of evidence support this KER for developmental vascular toxicity: (i) spatial correlation between altered vascular patterning and dysmorphogenesis; and (ii) concentration-dependent developmental toxicity with known anti-angiogenic compounds. Therapontos et al. [2009] determined that loss of immature blood vessels was the primary cause of thalidomide-induced teratogenesis in the chick limb, an effect phenocopied by anti-angiogenic but not anti-inflammatory metabolites/analogues of thalidomide. The thalidomide analog CPS49 suppressed chick limb-bud outgrowth only when the vasculature was at an immature stage of development; CPS49 did not suppress limb development post-innervation [Mahony et al. 2018]. Eight mechanistically diverse angiogenesis inhibitors (sunitinib, sorafenib, TNP-470, axitinib, pazopanib, vandetanib, everolimus, CPS49) suppressed

vascularization and invoked dysmorphogenesis in a concentration-dependent manner in both the chick limb-bud and zebrafish embryo models [Beedie et al. 2016]. Vatalanib, a selective VEGFR2 antagonist, suppressed vascular development in zebrafish embryos at 0.07 µM leading to vascular insufficiency by 72 hours post-fertilization (hpf), foreshadowing dysmorphogenesis at 0.22 µM by 120 hpf reduced survival of 10-day adults at 0.70 µM [Tal et al. 2014]. A tiered study evaluated two anti-angiogenic agents, 5HPP-33, a synthetic Thalidomide analog [Noguchi et al. 2005] and TNP-470, a synthetic Fumagillan analog [Ingber et al. 1990] across several complex *in vitro* functional assays: rat aortic explant assay, rat whole embryo culture, and zebrafish embryotoxicity [Ellis-Hutchngs et al. 2017]. Both compounds disrupted angiogenesis and embryogenesis but with modal differences: 5HPP-33 was embryolethal, and TNP-470 dysmorphic. The former blocks tubulin polymerization [Yeh et al. 2000; Inatsuki et al. 2005; Kizaki et al. 2008; Rashid et al. 2015] and the latter is a methionine aminopeptidase II inhibitor that suppresses non-canonical Wnt signals for endothelial proliferation [Ingber et al. 1990]. Transcriptomic profiles of exposed embryos pathways unique to each and in common to both, strongest being the TP53 pathway [Saili et al. 2019]. In mouse, TNP-470 reduced fetal intraocular microvasculature and induced microphthalmia [Rutland et al. 2009], which is a TP53-dependent phenotype [Wubah et al. 1996].

Uncertainties and Inconsistencies

Uncertainties and Inconsistencies: The cellular basis of tissue damage linked to vascular insufficiency is not well and represents a gap in understanding. During limb development, programmed cell death (PCD) contributes to separation of the digits. The onset of PCD is preceded by a genetically programmed increase of vascular density that directly determines with the extent of PCD and oxygen-dependent generation of reactive oxygen species (ROS) [Eshkar-Oren et al. 2015]. While many human and animal phenotypes associate with genetic signals and responses that control circulatory development, the causal relationship between vascular insufficiency and dysmorphogenesis is less understood due to various modes of tissue damage that may follow insufficient blood support (e.g., slow or weak heartbeat, poor vascularization, vessel occlusion, or reperfusion injury).

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