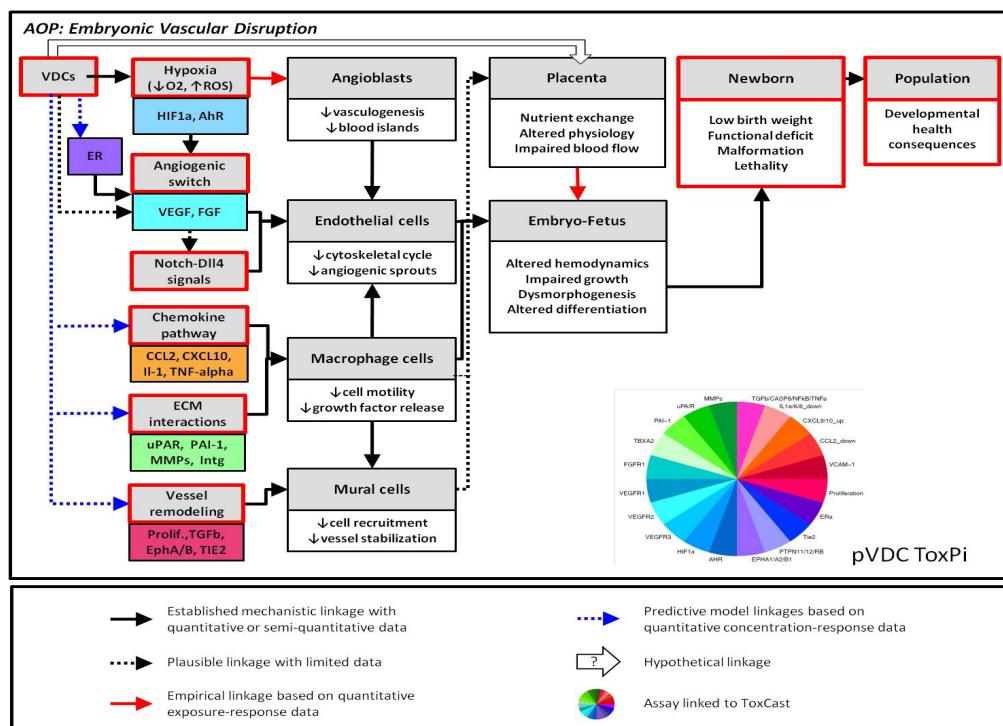


AOP 43: Disruption of VEGFR Signaling Leading to Developmental Defects

Short Title: Developmental Vascular Toxicity

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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, angiodyplasias 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.

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 (https://aopwiki.org/events/305)	Inhibition, VegfR2

Sequence	Type	Event ID	Title	Short name
2	KE	28	Reduction, Angiogenesis (https://aopwiki.org/events/28)	Reduction, Angiogenesis
3	KE	110	Impairment, Endothelial network (https://aopwiki.org/events/110)	Impairment, Endothelial network
4	KE	298	Insufficiency, Vascular (https://aopwiki.org/events/298)	Insufficiency, Vascular
5	AO	1001	Increased, Developmental Defects (https://aopwiki.org/events/1001)	Increased, Developmental Defects

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, VegfR2 (https://aopwiki.org/relationships/335)	adjacent	Reduction, Angiogenesis	High	High
Reduction, Angiogenesis (https://aopwiki.org/relationships/36)	adjacent	Impairment, Endothelial network	High	Moderate
Impairment, Endothelial network (https://aopwiki.org/relationships/125)	non-adjacent	Insufficiency, Vascular	Moderate	Low
Insufficiency, Vascular (https://aopwiki.org/relationships/1036)	non-adjacent	Increased, Developmental Defects	High	Moderate

Stressors

Name	Evidence
Vatalanib	
Sunitinib malate Sunitinib (INN)	

Overall Assessment of the AOP

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-1 β + TNF α + IFN γ); CASM3C (primary human coronary artery smooth muscle cells stimulated with IL-1 β + TNF α + IFN γ); LPS (HUVECs co-cultured with monocytes and stimulated with bacterial endotoxin); and hDFCGF (human dermal fibroblasts stimulated with IL-1 β + TNF α + IFN γ 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 TNF α 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). TNF α 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 (IL1 α , a macrophage-derived activator of TNF α) 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 IFN γ) 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 (ER α), also a trans-activator of VEGF expression. This included human ER α binding activity (NovaScreen), ER α 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 [Kleinsteuer 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-1 β + TNF α + IFN γ); and KF3T (human keratinocytes + fibroblasts stimulated with IL-1 β + TNF α + IFN γ + 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- β), 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.

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 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rats	Rattus norvegicus	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

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

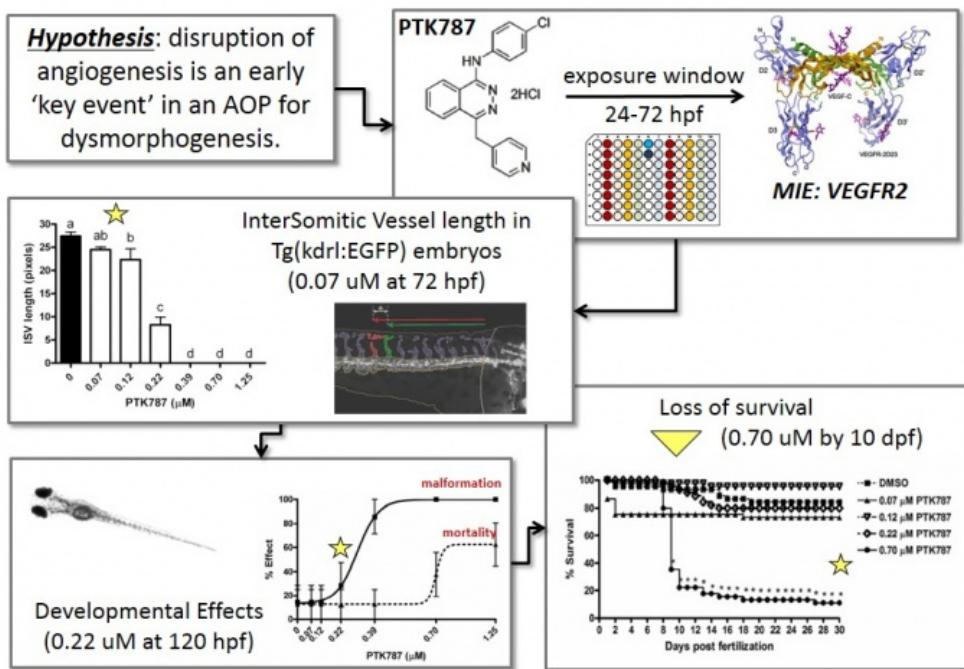
Essentiality of the Key Events

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

Weight of Evidence Summary

Weight of evidence for the MIE and AO are strong; the intermediate KEs have in some cases strong evidence but in other cases weaker evidence, due to the lack of quantitative information. The KERs are biologically plausible. Several manuscripts have been submitted or are in preparation that will help bolster weight of evidence [Belair et al. 2016; Nguyen et al. 2016; Tal et al. 2016; McCollum et al. 2016; Ellis-Hutchings et al. 2016; Franzosa et al. 2016; Knudsen et al. 2016].

Quantitative Consideration



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

(https://aopwiki.org/wiki/index.php/File:AOP_ZFE.jpg)

References

Ellis-Hutchings RG, Settivari RS, McCoy AT, Kleinstreuer N, Franzosa J, Knudsen TB and Carney EW. Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences. 2016 (in preparation).

Franzosa JA, Settivari RS, Ellis-Hutchings RG, Kleinstreuer NC, Houck KA, Carney EW and Knudsen TB. RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences. 2016 (in preparation).

Gold NB, Westgate MN, Holmes LB. Anatomic and etiological classification of congenital limb deficiencies. *American journal of medical genetics Part A*. 2011;155A(6):1225-35.

Houck KA, Dix DJ, Judson RS, Kavlock RJ, Yang J, Berg EL. Profiling bioactivity of the ToxCast chemical library using BioMAP primary human cell systems. *Journal of biomolecular screening*. 2009 Oct;14(9):1054-66. PubMed PMID: 19773588.

Husain T, Langlois PH, Sever LE, Gambello MJ. Descriptive epidemiologic features shared by birth defects thought to be related to vascular disruption in Texas, 1996-2002. *Birth defects research Part A, Clinical and molecular teratology*. 2008;82(6):435-40.

Kleinstreuer NC, Yang J, Berg EL, Knudsen TB, Richard AM, Martin MT, et al. Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nat Biotechnol*. 2014 Jun;32(6):583-91. PubMed PMID: 24837663.

Kleinstreuer NC, Dix D, Rountree M, Baker NC, Sipes NS, Reif D, et al. A computational model predicting disruption of blood vessel development. *PLoS computational biology*. 2013;9(4):e1002996.

Kleinstreuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, et al. Environmental impact on vascular development predicted by high-throughput screening. *Environmental health perspectives*. 2011;119(11):1596-603.

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C, Embryo today : reviews*. 2011;93(4):312-23.

Knudsen TB, Tomela T, Sarkkanen R, Spencer RS, Baker NC, Franzosa J, Tal T, Kleinstreuer NC, Cai B, Berg EL and Heinonen. ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models. 2016 (in preparation).

Kunkel EJ, Plavec I, Nguyen D, Melrose J, Rosler ES, Kao LT, et al. Rapid structure-activity and selectivity analysis of kinase inhibitors by BioMAP analysis in complex human primary cell-based models. *Assay and drug development technologies*. 2004 Aug;2(4):431-41. PubMed PMID: 15357924.

Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis*. 2005;8(1):43-51. PubMed PMID: 16132617.

McCollum CW, Vancells JC, Hans C, Vazquez-Chantada M, Kleinstreuer N, Tal T, Knudsen T, Shah SS, Merchant FA, Finnell RH, Gustafsson JA, Cabral R and Bondesson M. Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells. 2016 (in preparation).

Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Experimental cell research. 2002 Oct 15;280(1):119-33. PubMed PMID: 12372345.

Nguyen EH, Daly WT, Le NNT, Belair DG, Schwartz MP, Lebakken CS, Ananiev GE, Saghiri A, Knudsen TB, Sheibani N and Murphy WL. Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds. 2016 (in preparation).

Sarkanen JR, Mannerstrom M, Vuorenpa H, Uotila J, Ylikomi T, Heinonen T. Intra-Laboratory Pre-Validation of a Human Cell Based in vitro Angiogenesis Assay for Testing Angiogenesis Modulators. Frontiers in pharmacology. 2010;1:147.

Shirinifard A, McCollum CW, Bolin MB, Gustafsson JA, Glazier JA, Clendenon SG. 3D quantitative analyses of angiogenic sprout growth dynamics. Developmental dynamics : an official publication of the American Association of Anatomists. 2013;242(5):518-26.

Sipes NS, Martin MT, Kothiyal P, Reif DM, Judson RS, Richard AM, et al. Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays. Chem Res Toxicol. 2013 Jun 17;26(6):878-95. PubMed PMID: 23611293. Pubmed Central PMCID: 3685188.

Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S and Hemmer MJ. Immediate and long-term consequences of vascular toxicity during zebrafish development. Reproductive Toxicology. 2014;48:51-61.

Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum C, Bondesson M, Knudsen T, Padilla S and Kleinstreuer N. Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity. Reprod Toxicol (submitted).

Appendix 1

List of MIEs in this AOP

Event: 305: Inhibition, VegfR2 (<https://aopwiki.org/events/305>)

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 (https://aopwiki.org/aops/43)	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/PDGFR β /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 μ M and 0.077 μ M, 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 μ M in human endothelial cells (HUVECs) and concentration-dependent suppression of endothelial migration and tumorigenic formation of microvessels [Wood et al. 2000].

Evidence that this MIE 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 μ M 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 the MIE in the embryo.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra fish	Danio rerio	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

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 this MIE, VEGFR2 inhibition. Direct evidence for this comes from the susceptibility of vascular development to pharmacological inhibitors of human VEGFR2 kinase activity. Vatalanib (PTK787), for example, is a potent inhibitor of human VEGFR2 kinase activity [Wood et al. 2002] and disrupted angiogenic vessel formation in early zebrafish embryos at submicromolar concentrations [Tal et al. 2014].

Key Event Description

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

Vascular endothelial growth factor-A (VEGF-A), in particular the VEGF165 splice variant, plays a key role in the regulation of angiogenesis during early embryogenesis. This is evidenced by immature blood vessel formation and embryonic lethality in mutant mouse embryos heterozygous for the Vegfa-null allele [Ferrara et al. 1996; Carmellet et al. 1996]. VEGF-A is a soluble protein that acts directly on endothelial cells through two receptor tyrosine kinases: VEGFR1 (Flt-1) and VEGFR2 (KDR). The former is a decoy receptor that traps VEGF-A into corridors preventing interaction with the active receptor, VEGFR2. When liganded, VEGFR2 induces endothelial tip cell proliferation, survival, and vascular permeability. The specific aspect of the MIE considered here would involve factors leading to a change in VEGF-A gradients. This could include a change in the local production of VEGF-A, an increase in the decoy receptor (VEGFR1), or a drop in the expression or activity of VEGFR2. Chemical effects may commence at VEGF receptors (VEGFRs) by influencing local VEGF-A ligand production, ligand binding, receptor tyrosine kinase activity, or crosstalk with angiogenic chemokines, cytokines and growth factors. VEGF-A is locally produced in the vicinity of target

endothelial cells. Hypoxia (and chemical hypoxia) increases VEGF-A production through the HIF-alpha transcription pathway. VEGF-A can be trapped in the extracellular milieu by binding to components in the extracellular matrix (ECM) or VEGFR1. Since VEGFR1 binds VEGF-A with 10-fold greater affinity than does VEGFR2, VEGF-A is liberated by ECM breakdown during morphogenetic remodeling of tissues or matrix metalloproteinase (MMP) production during chemical injury. These events can positively or negatively regulate the local bioavailability of VEGF-A to its cognate receptor.

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

How it is Measured or Detected

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

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

Analysis of VEGFR2 expression under different physiological and toxicological contexts can be determined by standard array-based assays to define regulation of the angiogenic transcriptome, as well as targeted non-array methods to characterize cell-specific profiles during *in vivo* and *in vitro* development [Dumont et al. 1995; Abbott et al. 2000; Drake et al. 2007; Murakami et al. 2011]. In addition, the ontogenetic profile of VEGFR1 and VEGFR2 expression can be mapped in reporter zebrafish embryos under specific control of Flk or Flt gene regulatory elements [Tal et al. 2014]. Zebrafish possess 72 orthologs for 70% of human genes and 86% of 1318 human drug targets. Transgenic zebrafish that express reporter gene in developing blood vessels are fit for purpose to: visualize blood vessel formation during early development; localize and quantitate regional effects of chemicals on a phenotypic readout of angiogenic vessel formation; assess the reproducibility of vascular disruption across species; and evaluate the half-maximal inhibitory concentration (AC50) from a concentration-response curve.

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

References

Argraves WS, Larue AC, Fleming PA, Drake CJ. VEGF signaling is required for the assembly but not the maintenance of embryonic blood vessels. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2002;225(3):298-304.

Belair D, Schwartz MP, Knudsen T and Murphy WL. Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays. *Acta Biomaterialia* 2016. (in press).

Bhattacharya R1, Kwon J, Li X, Wang E, Patra S, Bida JP, Bajzer Z, Claesson-Welsh L and Mukhopadhyay D (2009) Distinct role of PLCbeta3 in VEGF-mediated directional migration and vascular sprouting. *J Cell Sci.* 122: 1025-1034.

Carmellet P, Ferreira V, Breier G, Pollefeyt S, Kleckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawliling J, Moons L, Collen D, Resau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435-439.

Chan J, Bayliss PE, Wood JM, Roberts TM (2002) Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell.* 1: 257-267. (Note: this paper describes the use of Vatalanib to inhibit VEGFR; PTK787 is a synonym for Vatalanib.)

Chappell JC, Taylor SM, Ferrara N, Bautch VL. Local guidance of emerging vessel sprouts requires soluble Flt-1. *Developmental cell.* 2009;17(3):377-86.

Chen DB, Zheng J. Regulation of placental angiogenesis. *Microcirculation* (New York, NY : 1994). 2014;21(1):15-25. Douglas NC, Tang H, Gomez R, Pytowski B, Hicklin DJ, Sauer CM, et al. Vascular endothelial growth factor receptor 2 (VEGFR-2) functions to promote uterine decidual angiogenesis during early pregnancy in the mouse. *Endocrinology.* 2009;150(8):3845-54.

Dorrell MI, Aguilar E, Friedlander M. Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocytic template and specific R-cadherin adhesion. *Investigative ophthalmology & visual science.* 2002;43(11):3500-10.

Eichmann A, Thomas JL. Molecular parallels between neural and vascular development. *Cold Spring Harbor perspectives in medicine.* 2013;3(1):a006551. Habeck H, Odenthal J, Walderich B, Maischein H, Schulte-Merker S. Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Current biology : CB.* 2002;12(16):1405-12.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* 380: 439-442.

Fischer et. al (2008) *Nat Rev Cancer* 8: 942-956.

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376: 66-70.

Gustafsdottir SM, Wennstrom S, Fredriksson S, Schallmeiner E, Hamilton AD, Sebti SM and Landegren U (2008) Use of proximity ligation to screen for inhibitors of interactions between vascular endothelial growth factor A and its receptors. *Clinical Chem* 54: 1218-1225.

Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci USA* 95: 9349-9354.

Hogan KA, Ambler CA, Chapman DL, Bautch VL. The neural tube patterns vessels developmentally using the VEGF signaling pathway. *Development* (Cambridge, England). 2004;131(7):1503-13.

Jang GH, Park IS, Lee SH, Huh TL, Lee YM. Malachite green induces cardiovascular defects in developing zebrafish (*Danio rerio*) embryos by blocking VEGFR-2 signaling. *Biochemical and biophysical research communications.* 2009;382(3):486-91.

Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW and Thomas KA (1999) *J Biol Chem* 274: 6453-6460.

Kleinsteuer N, Dix D, Rountree M, Baker N, Sipes N, Reif D, Spencer R and Knudsen T (2013) A computational model predicting disruption of blood vessel development. *PLoS Comp Biol* 9(4): 1-20. e1002996.

Kleinsteuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, DeWoskin R, Dix D, Kavlock R and Knudsen TB (2011) Environmental impact on vascular development predicted by high-throughput screening. *Environmental Hlth Persp* 119: 1596-1603.

Kleinsteuer N, Yang J, Berg E, Knudsen T, Richard A, Martin M, Reif D, Judson R, Polokoff M, Kavlock R, Dix D and Houck K (2014) Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nature Biotech* 32: 583-591.

Knudsen TB, Houck K, Sipes NS, Judson RS, Singh AV, Weissman A, Kleinsteuer NC, Mortensen H, Reif D, Setzer RW, Martin MT, Richard A, Dix DJ, and Kavlock RJ (2011) Activity profiles of 320 ToxCast™ chemicals evaluated Across 292 biochemical targets. *Toxicology* 282: 1-15.

Knudsen TB, Kleinsteuer NC (2011). Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C* 93: 312-323.

Knudsen TB, Kleinsteuer NC, Heinonen T (2014) Adverse Outcome Pathway for Embryonic Vascular Disruption: ToxCast HTS predictive model qualified by a validated human angiogenesis assay. Abstract presented at ToxCast Data Summit, manuscript in preparation.

Ligi I, Simoncini S, Tellier E, Grandvullemain I, Marcelli M, Bikfalvi A, et al. Altered angiogenesis in low birth weight individuals: a role for anti-angiogenic circulating factors. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet.* 2014;27(3):233-8.

Liu H, Yang Q, Radhakrishnan K, Whitfield DE, Everhart CL, Parsons-Wingerter P, et al. Role of VEGF and tissue hypoxia in patterning of neural and vascular cells recruited to the embryonic heart. *Developmental dynamics : an official publication of the American Association of Anatomists.* 2009;238(11):2760-9.

Nimmagadda S, Geetha Loganathan P, Huang R, Scaal M, Schmidt C, Christ B. BMP4 and noggin control embryonic blood vessel formation by antagonistic regulation of VEGFR-2 (Quk1) expression. *Developmental biology.* 2005;280(1):100-10.

Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *The American journal of pathology.* 2004;164(5):1531-5.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.

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Shibuya M (2013) Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem*. 153: 13-19.

Shirinifard A, McCollum CW, Bolin MB, Gustafsson JA, Glazier JA, Clendenon SG. 3D quantitative analyses of angiogenic sprout growth dynamics. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2013;242(5):518-26.

Sipes NS, Martin MT, Kothiyal P, Reif DM, Judson R, Richard A, Houck KA, Dix DJ, Kavlock RJ and Knudsen TB (2013) Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays *Chem Res Toxicol* 26: 878-895.

Stankunas K, Ma GK, Kuhnert FJ, Kuo CJ, Chang CP. VEGF signaling has distinct spatiotemporal roles during heart valve development. *Developmental biology*. 2010;347(2):325-36.

Tal T, Kleinstreuer N, Toimela T, Sarkkanen R, Heinonen T, Knudsen T and Padilla S (2014) Identification of chemical vascular disruptors during development using an integrative predictive toxicity model and zebrafish and in vitro functional angiogenesis assays. Abstract presented at ToxCast Data Summit, manuscript in preparation.

Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S and Hemmer MJ (2014) Immediate and long-term consequences of vascular toxicity during zebrafish development. *Reproductive Toxicology* 48:51-61.

van den Akker NM, Molin DG, Peters PP, Maas S, Wisse LJ, van Brempt R, et al. Tetralogy of fallot and alterations in vascular endothelial growth factor-A signaling and notch signaling in mouse embryos solely expressing the VEGF120 isoform. *Circulation research*. 2007;100(6):842-9.

Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood*. 2006;107(3):931-9.

Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Metz H, O'Reilly T, Persohn E, Rösel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirmer M, Thierauch KH, Schneider MR, Dreys J, Martiny-Baron G, Totzke F (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res* 60: 2178-2189.

Xia M, Bi K, Huang R, Cho MH, Sakamuru S, Miller SC, et al. Identification of small molecule compounds that inhibit the HIF-1 signaling pathway. *Molecular cancer*. 2009;8:117.

Yabu T, Tomimoto H, Taguchi Y, Yamaoka S, Igarashi Y, Okazaki T. Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate. *Blood*. 2005;106(1):125-34.

List of Key Events in the AOP

Event: 28: Reduction, Angiogenesis (<https://aopwiki.org/events/28>)

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 (https://aopwiki.org/aops/43)	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 transgenic zebrafish embryos [Tal et al. 2016].

Belair et al. [2016] designed and characterized a chemically human angiogenesis iPSC-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. 2016]. This approach revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species. Homology appeared first in the receptor tyrosine kinase signaling systems, followed in turn by the urokinase plasminogen activating (uPA) receptor (uPAR) system and chemokine/G-protein coupled receptor system.

Key Event Description

Developmental angiogenesis involves a complex interplay between the native extracellular matrix, vascular endothelial cells (EC), growth factors, and cytokines/chemokines [Knudsen and Kleinstreuer, 2011]. In vitro models are presently used to study EC function and screen for angiogenesis inhibitors based on effects on cell proliferation, sprouting behavior, and tubulogenesis. Sprouting is driven by matrix metalloproteinase (MMP) activity whereas tubulogenesis shows MMP-dependence only in three-dimensional (3D) contexts. Furthermore, the VEGF-dependence is unclear in some tubulogenesis assay platforms and this further limits comparisons of EC tubulogenesis to VEGF-dependent vascular formation in vivo. As such, EC sprouting models that recapitulate MMP-dependent and VEGF-dependent endothelial cell invasion provide a more physiologic context for modeling early stages of angiogenesis and can be used to evaluate the extracellular matrix (ECM) degradation and invasion characteristic of angiogenic sprouting in vivo [Belair et al. 2016].

How it is Measured or Detected

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

References

Belair DG, Schwartz MP, Knudsen TB, Murphy WL. Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays. *Acta Biomaterialia* 2016 (in press).

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C, Embryo today: reviews*. 2011 Dec;93(4):312-23. PubMed PMID: 22271680.

Lamalice L, Le Boeuf F and Huot J. Endothelial cell migration during angiogenesis. *Circ Res*. 2007 Mar 30;100(6):782-94.

Eilken HM and Adams RH. Dynamics of endothelial cell behavior in sprouting angiogenesis. *Curr Opin Cell Biol*. 2010 Oct;22(5):617-25. doi: 10.1016/j.ceb.2010.08.010.

Shirinifard A, McCollum CW, Bondesson MB, Gustafsson JA, Glazier JA and Clendenon SG. 3D Quantitative analyses of angiogenic sprout growth dynamics *Devel Dynam*. 2013 242(5): 518-526.

Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum C, Bondesson M, Knudsen T, Padilla S and Kleinstreuer N. Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity. *Reprod Toxicol* (submitted).

Event: 110: Impairment, Endothelial network (<https://aopwiki.org/events/110>)

Short Name: Impairment, Endothelial network

Key Event Component

Process	Object	Action
endothelium development		abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	KeyEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF (https://aopwiki.org/aops/150)	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 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Blood vessel development utilizes highly conserved molecular pathways that are active across vertebrate species. Anatomically, however, the molecular toolbox for vasculogenesis/angiogenesis has varied themes for arterial, venous, and lymphatic channels, as well as across different organs and species [Tal et al. 2016]. ToxCast high-throughput screening (HTS) data for 25 assays mapping to targets in embryonic vascular disruption signature [Knudsen and Kleinstreuer, 2011] were used to rank-order 1060 chemicals for their potential to disrupt vascular development. The predictivity of this signature is being evaluated in various angiogenesis assays, including tubulogenesis in endothelial cells from zebrafish, chick, mouse and human species [Tal et al. 2016; Vargesson et al. 2003; Knudsen et al. 2016; McCollum et al. 2016; Nguyen et al. 2016]. As an example, a zebrafish embryo vascular model in conjunction with a mouse endothelial cell model identified 28 potential vascular disruptor compounds (pVDCs) from ToxCast. These exposures invoked a plethora of vascular perturbations in the zebrafish embryo, including malformed intersegmental vessels, uncondensed caudal vein plexus, hemorrhages and cardiac edema; 22 pVDCs inhibited endothelial tubulogenesis in an yolk-sac-derived endothelial cell line [McCollum et al. 2016]. The VEGF pathway was implicated across mouse-zebrafish species. Because gene sequence similarity of the ToxCast pVDC signature is comprised of proteins that primarily map to human in vitro and biochemical assays, the U.S. EPA SeqAPASS tool was used to assess the degree of conservation of signature targets between zebrafish and human, as well as other commonly used model organisms in human health and environmental toxicology research [Tal et al. 2016]. This approach revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species.

Key Event Description

In embryological terms the angiogenic cycle entails a stepwise progression of de novo blood vessel morphogenesis (vasculogenesis), maturation and expansion (angiogenesis), and remodeling [Hanahan, 1997; Chung and Ferrara 2011; Coulter et al. 2005]. These events commence as angioblasts migrate, proliferate, and assemble into a tubular network. With maturation, the endothelial tubules co-opt local stromal cells as

pericytes and smooth muscle. Local signals acting on receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and glycosyl phosphatidyl-inositol (GPI)-anchored receptors, and later vascular flow-mediated signals. The process of endothelial assembly into a tubular network may be disrupted by environmental agents [Sarkanen et al. 2010; Bondesson et al. 2016; Knudsen et al. 2016; Nguyen et al. 2016; Tal et al. 2016].

How it is Measured or Detected

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

References

Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NFK and Wheatley DN. An *in vitro* model of angiogenesis: Basic features. *Angiogenesis*. 1999 3(4): 335-344.

Chung AS, Ferrara N. Developmental and pathological angiogenesis. *Annual review of cell and developmental biology*. 2011;27:563-84. PubMed PMID: 21756109.

Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005 Dec 15;438(7070):937-45. PubMed PMID: 16355211.

Hanahan D. Signaling vascular morphogenesis and maintenance. *Science*. 1997 Jul 4;277(5322):48-50. PubMed PMID: 9229772.

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C, Embryo today : reviews*. 2011;93(4):312-23.

Knudsen TB, Tomela T, Sarkanen R, Spencer RS, Baker NC, Franzosa J, Tal T, Kleinstreuer NC, Cai B, Berg EL and Heinonen. ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models. 2016 (in preparation).

Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis*. 2005;8(1):43-51. PubMed PMID: 16132617.

McCollum CW, Vancells JC, Hans C, Vazquez-Chantada M, Kleinstreuer N, Tal T, Knudsen T, Shah SS, Merchant FA, Finnell RH, Gustafsson JA, Cabrera R and Bondesson M. Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells. 2016 (in preparation).

Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. *Experimental cell research*. 2002 Oct 15;280(1):119-33. PubMed PMID: 12372345.

Nguyen EH, Daly WT, Le NNT, Belair DG, Schwartz MP, Lebakken CS, Ananiev GE, Saghiri A, Knudsen TB, Sheibani N and Murphy WL. Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds. 2016 (in preparation).

Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum C, Bondesson M, Knudsen T, Padilla S and Kleinstreuer N. Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity. *Reprod Toxicol* (submitted).

Vargesson N. Vascularization of the developing chick limb bud: role of the TGF β signalling pathway. *J Anat*. 2016 Jan, 202(1): 93-103. PMCID: PMC1571066.

Event: 298: Insufficiency, Vascular (<https://aopwiki.org/events/298>)

Short Name: Insufficiency, Vascular

Key Event Component

Process	Object	Action
blood circulation	blood	decreased
	capillary plexus	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:43 - Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	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. 2016; Franzosa et al. 2016].

Key Event Description

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

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

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

How it is Measured or Detected

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

References

Chung AS, Ferrara N. Developmental and pathological angiogenesis. Annual review of cell and developmental biology. 2011;27:563-84. PubMed PMID: 21756109.

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Clendenon SG, Sankaran DG, Shirinifard A, McCollum CW, Bondesson MB, Gustafsson JA and Glazier JA. Arsenic exposure inhibits angiogenesis in zebrafish via downregulation of both VEGFA and VEGFR2. *Microscopy and Microanalysis*. 2013;19(S2): 778-779.

Ellis-Hutchings RG, Settivari RS, McCoy AT, Kleinstreuer N, Franzosa J, Knudsen TB and Carney EW. Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences. 2016 (in preparation).

Eshkar-Oren I, Krief S, Ferrara N, Elliott AM, Zelzer E. Vascular patterning regulates interdigital cell death by a ROS-mediated mechanism. *Development* (Cambridge, England). 2015 Feb 15;142(4):672-80. PubMed PMID: 25617432.

Franzosa JA, Settivari RS, Ellis-Hutchings RG, Kleinstreuer NC, Houck KA, Carney EW and Knudsen TB. RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences. 2016 (in preparation).

Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development*. 2005;132: 5199-209.

Kleinstreuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, et al. Environmental impact on vascular development predicted by high-throughput screening. *Environmental health perspectives*. 2011 Nov;119(11):1596-603. PubMed PMID: 21788198. Pubmed Central PMCID: PMC3226499.

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C, Embryo today : reviews*. 2011 Dec;93(4):312-23. PubMed PMID: 22271680.

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature*. 1997 Mar 27;386(6623):403-7. PubMed PMID: 9121557.

McCollum CW, Vancells JC, Hans C, Vazquez-Chantada M, Kleinstreuer N, Tal T, Knudsen T, Shah SS, Merchant FA, Finnell RH, Gustafsson J-A, Cabrera R and Bondesson M (2016) Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells. (submitted).

Shirinifard A, McCollum CW, Bondesson MB, Gustafsson JA, Glazier JA and Clendenon SG. 3D Quantitative analyses of angiogenic sprout growth dynamics *Devel Dynam*. 2013;242(5): 518-526.

Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum C, Bondesson M, Knudsen T, Padilla S and Kleinstreuer N (2016) Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity. (submitted).

Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S and Hemmer MJ. Immediate and long-term consequences of vascular toxicity during zebrafish development. *Reproductive Toxicology*. 2014;48:51-61.

Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 May 26;106(21):8573-8. PubMed PMID: 19433787. Pubmed Central PMCID: 2688998.

Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, et al. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer research*. 2007;67: 11386-92.

van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N. Teratogenic mechanisms of medical drugs. *Human reproduction update*. 2010 Jul-Aug;16(4):378-94. PubMed PMID: 20061329.

Vargesson N. Thalidomide-induced teratogenesis: history and mechanisms. *Birth defects Research Part C, Embryo today: reviews*. 2015 Jun;105(2):140-56. PubMed PMID: 26043938.

Yozzo KL, Isales GM, Raftery TD, Volz DC. High-content screening assay for identification of chemicals impacting cardiovascular function in zebrafish embryos. *Environmental science & technology*. 2013;47: 11302-10.

List of Adverse Outcomes in this AOP

Event: 1001: Increased, Developmental Defects (<https://aopwiki.org/events/1001>)

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 (https://aopwiki.org/aops/43)	AdverseOutcome

Biological Context

BIOLOGICAL CONTEXT

Level of Biological Organization

Molecular

Domain of Applicability

Wilson's Principles of Teratology (circa 1977) support the taxonomic applicability of AO1001. According to these long-standing Wilson's principles, the first on "Susceptibility to Teratogenesis Depends on the Genotype of the Conceptus and a Manner in which this Interacts with Adverse Environmental Factors". This principle has four main tenets:

- i) species differences account for the fact that certain species respond to particular teratogens where others do not, or at least not to the same extent (e.g., humans and other primates are vulnerable to thalidomide induced phocomelia whereas rodents are not);
- ii) strain and intralitter differences account for the fact that some lineages of the same species with different genetic backgrounds can differ in teratogenic susceptibility;
- iii) gene-environment interplay results in different patterns of abnormalities between organisms with the same genome raised in different environments, and between organisms with different genomes raised in the same environment; and
- iv) multifactorial causation accounts for the complex interactions involving more than one gene and/or more than one environmental factor.

Key Event Description

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

AO1001 consolidates into one, the four main types of developmental defects observed in regulatory guideline studies (prenatal loss, malformations, low birth weight, and postnatal function). Any or all of these developmental defects may occur within the same litter or study. Congenital malformations refer to alterations in normal development that result from intrinsic (genetically programmed) or extrinsic (environmentally induced) perturbations of development. Mechanistically, some pathways may lead to specific types of malformations; however, a fundamental principle is that all four types of endpoints are important for hazard assessment. This is because even a simple MIE may disrupt the embryo in multiple ways that depend on the nature of the insult and timing of exposure.

How it is Measured or Detected

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

References

Friedman JM. The principles of teratology: are they still true? *Birth Defects Res A*. 2010 Oct;88(10):766-8. doi: 10.1002/bdra.20697.

Janer G, Slob W, Hakkert BC, Vermeire T and Piersma AH. A retrospective analysis of developmental toxicity studies in rat and rabbit: what is the added value of the rabbit as an additional test species? *Regul Toxicol Pharmacol*. 2008 50: 206-217.

Hurt ME, Cappon GD and Browning A. Proposal for a tiered approach to developmental toxicity testing for veterinary pharmaceutical products for food-producing animals. *Food Chem Toxicol*. 2003 41: 611-619.

Knudsen TB, Martin MT, Kavlock RJ, Judson RS, Dix DJ and Singh AV. Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U.S. EPA's ToxRefDB. *Reprod Toxicol*. 2009 28: 209-219.

Rorije E, van Hienen FJ, Dang ZC, Hakkert BH, Vermeire T and Piersma AH. Relative parameter sensitivity in prenatal toxicity studies with substances classified as developmental toxicants. *Reprod Toxicol*. 2012 34: 284-290.

Schardein J. *Chemically Induced Birth Defects*. 2000. New York, Marcel Decker Inc.

Theunissen PT, Beken S, Beyer BK, Breslin WJ, Cappon GD, Chen C, Chmielewski G, De Schaeppdrijver L, Enright B, Foreman JE, Harrouk W,

Hew KW, Hoberman AM, Hui JY, Knudsen TB, Laffan SB, Makris S, Martin M, McNerney ME, Siezen CL, Stanislaus DJ, Stewart J, Thompson KE, Tornesi B, Weinbauer G, Wood S, Van der Laan JW and Piersma AH. Comparison of rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on the nature and severity of developmental effects. *Chem Rev Toxicol.* 2016 (in revision).

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 335: Inhibition, VegfR2 leads to Reduction, Angiogenesis (<https://aopwiki.org/relationships/335>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

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

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

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

Empirical Evidence

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

Uncertainties and Inconsistencies

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

References

Belair D, Schwartz MP, Knudsen T and Murphy WL. Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays. *Acta Biomaterialia* 2016. (in press).

Carmellet P, Ferreira V, Breier G, Pollefeyt S, Kleckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawlling J, Moons L, Collen D, Resau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435-439.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 380: 439-442.

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Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376: 66-70.

Herbert SP and Stainier DY. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol*. 2011 Aug 23;12(9):551-564. doi: 10.1038/nrm3176.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.

Shirinifard A, McCollum CW, Bolin MB, Gustafsson JA, Glazier JA, Clendenon SG. 3D quantitative analyses of angiogenic sprout growth dynamics. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2013;242(5):518-26.

Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S and Hemmer MJ (2014) Immediate and long-term consequences of vascular toxicity during zebrafish development. *Reproductive Toxicology* 48:51-61.

Relationship: 36: Reduction, Angiogenesis leads to Impairment, Endothelial network
(<https://aopwiki.org/relationships/36>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

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

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

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

Empirical Evidence

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

References

Belair D, Schwartz MP, Knudsen T and Murphy WL. Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays. *Acta Biomaterialia* 2016. (in press).

Kleinstreuer NC, Yang J, Berg EL, Knudsen TB, Richard AM, Martin MT, et al. Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nat Biotechnol*. 2014 Jun;32(6):583-91. PubMed PMID: 24837663.

McCollum CW, Vancells JC, Hans C, Vazquez-Chantada M, Kleinstreuer N, Tal T, Knudsen T, Shah SS, Merchant FA, Finnell RH, Gustafsson JA, Cabrera R and Bondesson M. Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells. 2016 (in preparation).

Nguyen EH, Daly WT, Le NNT, Belair DG, Schwartz MP, Lebakken CS, Ananiev GE, Saghiri A, Knudsen TB, Sheibani N and Murphy WL. Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds. 2016 (in preparation).

Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum C, Bondesson M, Knudsen T, Padilla S and Kleinstreuer N. Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity. *Reprod Toxicol* (submitted).

List of Non Adjacent Key Event Relationships

Relationship: 125: Impairment, Endothelial network leads to Insufficiency, Vascular
(<https://aopwiki.org/relationships/125>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	non-adjacent	Moderate	Low

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

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

Empirical Evidence

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

References

Gold NB, Westgate MN, Holmes LB. Anatomic and etiological classification of congenital limb deficiencies. American journal of medical genetics Part A. 2011 Jun;155A(6):1225-35. PubMed PMID: 21557466.

Husain T, Langlois PH, Sever LE, Gambello MJ. Descriptive epidemiologic features shared by birth defects thought to be related to vascular disruption in Texas, 1996-2002. Birth defects research Part A, Clinical and molecular teratology. 2008 Jun;82(6):435-40. PubMed PMID: 18383510.

Kleinstreuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, et al. Environmental impact on vascular development predicted by high-throughput screening. Environmental health perspectives. 2011 Nov;119(11):1596-603. PubMed PMID: 21788198. Pubmed Central PMCID: PMC3226499.

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. Birth defects research Part C, Embryo today : reviews. 2011 Dec;93(4):312-23. PubMed PMID: 22271680.

Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S and Hemmer MJ. Immediate and long-term consequences of vascular toxicity during zebrafish development. Reproductive Toxicology. 2014;48:51-61.

Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. Proceedings of the National Academy of Sciences of the United States of America. 2009 May 26;106(21):8573-8. PubMed PMID: 19433787. Pubmed Central PMCID: 2688998.

van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N. Teratogenic mechanisms of medical drugs. Human reproduction update. 2010 Jul-Aug;16(4):378-94. PubMed PMID: 20061329.

Relationship: 1036: Insufficiency, Vascular leads to Increased, Developmental Defects
(<https://aopwiki.org/relationships/1036>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Disruption of VEGFR Signaling Leading to Developmental Defects (https://aopwiki.org/aops/43)	non-adjacent	High	Moderate

Evidence Supporting this KER

Empirical Evidence

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

References

Gold NB, Westgate MN, Holmes LB. Anatomic and etiological classification of congenital limb deficiencies. American journal of medical genetics Part A. 2011 Jun;155A(6):1225-35. PubMed PMID: 21557466.

AOP43

Husain T, Langlois PH, Sever LE, Gambello MJ. Descriptive epidemiologic features shared by birth defects thought to be related to vascular disruption in Texas, 1996-2002. *Birth defects research Part A, Clinical and molecular teratology*. 2008 Jun;82(6):435-40. PubMed PMID: 18383510.

Kleinstreuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, et al. Environmental impact on vascular development predicted by high-throughput screening. *Environmental health perspectives*. 2011 Nov;119(11):1596-603. PubMed PMID: 21788198. Pubmed Central PMCID: PMC3226499.

Knudsen TB, Kleinstreuer NC. Disruption of embryonic vascular development in predictive toxicology. *Birth defects research Part C, Embryo today : reviews*. 2011 Dec;93(4):312-23. PubMed PMID: 22271680.

Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 May 26;106(21):8573-8. PubMed PMID: 19433787. Pubmed Central PMCID: 2688998.

van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N. Teratogenic mechanisms of medical drugs. *Human reproduction update*. 2010 Jul-Aug;16(4):378-94. PubMed PMID: 20061329.