Organisation for Economic Co-operation and Development

Adverse Outcome Pathway Scientific Review Report

AOP 43: Disruption of Vascular Endothelial Growth Factor Receptor (VEGFR) signaling leading to developmental defects

Short name: Developmental Vascular Toxicity

This document is the **scientific review report of AOP43,** elaborated after authors had worked on reviewers comments.

It has been prepared by Mr Jean-Baptiste FINI (fini@mnhn.fr), consultant for the OECD Secretariat.

It compiles the views and comments of the reviewers and explains how the authors of the AOP have addressed these comments.

It provides the basis to EAGMST for determining if AOP 43 has been adequately revised by their authors following the review and if it can be released to the Working Party of the National Coordinators of the Test Guidelines Programme and to the Working Party on Hazard Assessment for endorsement.

OECD contact:
Nathalie DELRUE : Nathalie.DELRUE@oecd.org

Table of contents

1. Introduction and background to AOP 43: Disruption of Vascular Endothelial Growth Factor Receptor (VEGFR) signaling leading to developmental defects

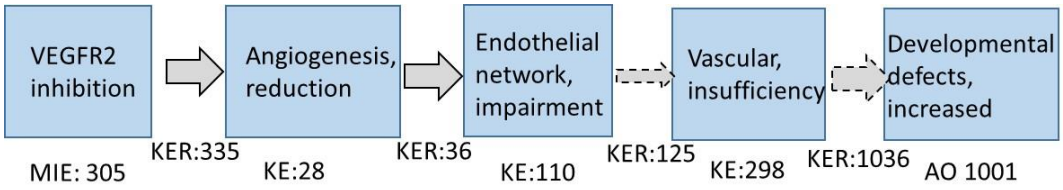
The cardiovascular system is the first functional organ system to develop in the vertebrate embryo, reflecting its critical role during normal development and pregnancy. Elucidating an AOP for embryonic vascular disruption must consider the stepwise events underlying blood vessel patterning. Vascular 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 remodelling. Critical pathways involve receptor tyrosine kinases (e.g., growth factor-signaling), G-protein coupled receptors (e.g., chemokine signalling), and glycosyl phosphatidyl inositol (GPI)-anchored receptors (e.g. uPAR system).

This AOP focuses on the regulation and disruption of vasculogenesis-angiogenesis during embryonic development via disruption of the VEGF-signalling pathway. VEGFA[1] 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 is the postulated molecular initiating event (MIE) of this AOP. Other initiating events (eg VEGFA production) may be included in new AOPs, developed and linked to this AOP in the future.
Downstream Key Events (KE) include altered cell fate and behaviour of "endothelial tip cells" (exploratory behaviour, 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 a MIE is invoked.

[1] VEGFA: Vascular Endothelial Growth Factor A

***Figure 1*: Graphical representation of AOP 43**

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



The bottom version is a suggestion made by the review panel and review manager, to better appreciate KER strength and adjacency. Size of the arrow indicates robustness and dashed lines show non adjacent Key Even.

1.1. AOP 43 authors

Seven authors participated in the development of AOP43:

- **Tom Knudsen** knudsen.thomas@epa.gov (corresponding author), Nancy Baker (Leidos) baker.nancy@epa.gov, Richard Spencer (GDIT) - spencer.richard@epa.gov - US EPA , National center for computational technology, research triangle park;
- Tuula Heinonen tuula.heinonen@uta.fi - Finnish Centre for Alternative Methods, University of Tampere, Tampere Finland;
- Rob Ellis-Hutchings rellis-hutchings@dow.com - The Dow Chemical Company, Midland MI, USA.
- Neil Vargesson – n.vargesson@abdn.ac.uk - University of Aberdeen, Aberdeen, Scotland UK.
- Nicole Kleinstreuer – nicole.kleinstreuer@nih.gov - National Toxicology Program/NICEATM-ICCVAM, Research Triangle Park NC, USA.

1.2. Scientific Reviewers

This AOP was reviewed in summer 2021 by a panel of three reviewers (see Annex 1). The panel was established based on nominations by the Working Party of the Test Guidelines Programme, following a request for nominations sent by the OECD Secretariat in November 2020. Attempt to broaden size of the panel failed since the number of experts in this field is limited. However, the collective expertise of the nominated panel, covering areas such as angiogenesis, developmental toxicology or VegF inhibition, was deemed sufficient by the review manager and the OECD Secretariat and the review started in May 2021.

2. Synthesis of main issues of the review

This section provides an overview of issues raised by the three members of the scientific reviewer panel (see Annex 1 for details on panel composition).

Reviewers were asked to reply to the following charge questions regarding different aspects of the AOP:

1. **Scientific quality:**
   - **Does the AOP incorporate the appropriate scientific literature?**
   - **Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?**

2. **Weight of evidence:**
   - **Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?**

3. **Additional observations:**
   - **What do you consider to be critical data gaps, if any, and how to fill these gaps?**

The version for review was the snapshot provided by the OECD Secretariat and accessible at https://aopwiki.org/aopwiki/snapshot/pdf_file/43-2021-02-23T17:25:31+00:00.pdf

A summary of the reviewers' answers to the charge questions for each point is accessible below, with quotations organised point by point under each question. The complete reviews, with planed modifications by the authors are accessible in Annex 2 of this report.

2.1. Scientific quality

Does the AOP incorporate the appropriate scientific literature?

All reviewers (R1, 2 and 3) agreed with the fact that this AOP needed more work before being endorsed. All reviewers agreed with the fact that literature was not up to date and asked for an update of the literature. Reviewers agreed on the quality of the writing of this AOP but asked for significant improvement with a few editorial elements to be checked.

Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

In general this point is satisfying. An update on stressors (R3) and literature (R1) is suggested.

2.2. Weight of evidence

Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

Key Events are well described and the vast majority of the KEs and KERs are correctly weighted. However, justifications are not present and some precisions are needed. Therefore reviewers felt that the weight of evidence was not appropriately described .

Some examples of comments from reviewers 1 and 3 are given below:

Overall AOP

R1_ No. The WoE is mostly lacking any description and justification. A summarizing table as suggested in the Users Handbook (p51) on direct or indirect evidence is missing. All sections regarding Quantitative Understanding are missing.

R2_No_inconsistent relationships described. Scoring not described or justified.

The specificity of the inhibitors includes activity on other receptors, eg. PDGFR

Key Events

MIE: 305_ R1: buildup of the AngioKB database should be removed as well as references to other pathways. It is mentioned that several assays directly measure capacity or bioactivity but only one example is given for each trait. Instead, all relevant assays measuring VEGFR2 capacity and bioactivity should be named directly.

Zebrafish genetic homology should be discussed

R2_**The supporting literature provided is rather old – PubMed results in ca 2700 articles, of which >260 reviews in the last 5 years**

Other questions about specificity of stressors and rationale behind choosing VEGFR2 over the other receptors

KE:28_ R1_Domain of Applicability, the text here is not relevant for this section and should be moved to other sections

p11, Key Event description needs more description to define this event and distinguish it from KE305 and KE110. In the abstract of the AOP measurable events like altered cell fate and behavior of tip and stalk cells are mentioned.

R2_ all subsections (DOA, Description, how it is measured) **need referencing**

KE:110_ R1-irrelevent DOA. Key Event description needs more description to define this event and distinguish it from KE305 and KE110

KE:298

R1_Key Event description needs careful revision, for better distinction from KE28. Differences of KE28 and KE110 should be discussed in the section for “Essentiality of KEs” of AOP43.

R2_irrevelent DOA

AO 1001

R1_Domain of Applicability is not relevant for this section and should be moved to other sections

Section for the "Regulatory Significance of the AO" is missing

R2_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

R3_Authors selected the four main types of developmental defects such as prenatal loss, malformations, low birth weight, and postnatal function. How did authors choose four events? Viability after delivery is also important event

Key Event Relationships

KER:335

R1_Missing sections: Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding)

KER:36

R1_Missing sections: Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding)

R2_Suggestion Changing name of KER. *It would be more accurate if the terminology were specific to* ***angiogenic sprouting****, in which case it may be OK*

KER:125

R1_ It should be considered either to change this KER to "Impairment, Endothelial Network lead to Insufficiency, Blood Flow" or to remove this KE altogether. Instead a KER "Impairment, Endothelial Network lead to Increased, Developmental Defects" could be created

R2_The description refers to the next KER (developmental defects), not KER 125

KER:1036

R1_Missing sections: KER Description, Biological Plausibility, Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding)

R2_**weak direct support**

2.3. Critical gaps in the AOP 43

- *What do you consider to be critical data gaps, if any, and how to fill these gaps*

In its original form, the AOP was judged difficult to use for regulatory purpose as the applicability is very narrow (VEGF inhibition). Authors made the point that the MIE was originally broader but was redefined following the internal review. The initial description involved various molecular signaling pathways and MIEs. The internal reviewers suggested to focus on VEGF signaling which seemed to be a node to which various AOPs converge and also suggested to potentially consider other MIE/KE/AO in the context of a network of AOPs.

The panel also noted that available data in the literature would benefit from being presented in a more structured way, demonstrating quantitative relationships of MIE leading to AO. Most KEs and KERs had essential sections missing completely or not used in alignment with the Users' Handbook.

According to this document, the Essentiality of the Key Events section is supposed to be organised in "tabular" form, but the authors opted for paragraph form. Ideally, this section would be re-organised in a table with clear, scientifically supported evidence statements (i.e., high, medium, low).

- *Additional observations*

Some comments were raised at the end of review teleconference on the domain of applicability (Embryonic? Developmental?).

2.4. Conclusion

Although this AOP was considered well written, the reviewers agreed that most of the sections needed a deep revision. A significant amount of work was required to improve the AOP before going forward in the endorsement process.

Authors were expected to reply to all reviewers' comments. Gaps identified by the reviewers are listed in section §2.2 and in Annex 2). The main revisions were discussed at the end of review TC with both authors and reviewers.

3. Summary record of the teleconference

3.1. TC agenda

Two teleconferences were organised during the summer/fall 2021.

1) Only reviewers and the review manager attended the first teleconference.
It took place beginning of August 2021 and aimed at defining the role of the reviewers and discuss the first comments reviewers had on the AOP, in particular the main issues the reviewers found important to ask the authors. All three reviewers attended the TC.

The individual comments from the three reviewers were collected and harmonised. An initial review report was written by the review manager and sent to the reviewers on the first week of November 2021.

2) The end-of-review teleconference was organised on December 14th 2021.
The AOP main authors, Tom Knudsen and Nancy Baker, the three reviewers and the review manager attended the TC.
The review manager and the authors thanked the reviewers who devoted significant amount of their time to provide constructive comments, editorial changes and additional literature, before the TC.

All the materials cited have been made available to the authors.
The authors replied to the principal comments raised by the reviewers before the TC but did not have time to provide a point by point rebuttal in case of disagreement with the comment, before the TC. Nevertheless, the authors agreed on all changes required by the reviewers and indicated that they would address them by February 2022.
The agenda of the TC was as follows:

- Brief reminders of what we can be expected from an AOP and what an AOP is not.
- Brief reminders on AOP 43, the different Key Events (KE) and Key Event Relationships (KER), the review process and questions asked to the reviewers.
- Discussion of issues raised by the reviewers and answers provided by the authors.
- Other Issues
- Conclusions and elements on the upcoming events in the AOP review/endorsement process.

3.2. Main issues and responses during the call

The main issue raised by the reviewers was that the scientific literature was not up to date.
Authors were asked to incorporate missing literature.

To address the general comment, Tom Knudsen and Nancy Baker defined a series of complex queries to mine the literature with the 'AbstractSifter' tool on November 30, 2021. This returned 169 PubMed records, of which 76 were cited previously and **93 were new.** The list was presented and is accessible in Annex 3. All these new articles were meant to be incorporated into AOP 43.

All Key Events and Key Events Relationships were discussed. Even though the structure of the AOP was acknowledged by the whole panel some questions still remained on the description of Key Events and Key Events Relationships. The sections “how it is measured or detected” needed also revisions.

The reviewers acknowledged the amount of work already done by the authors before the TC. However, another piece of work was required before going further in the process.

Finally, regarding the shortcomings of the AOP, the authors were asked to elaborate on the potential application of the AOP. The authors agreed to develop such section, recognising the use of this AOP, together with other AOPs being developed with same AO, as part of an IATA. An AOP network has been discussed by the authors and the panel but it was decided to do it once this AOP would be finalised and endorsed.

Overall, the authors agreed to implement the suggested changes by updating and making changes to specific sections of this AOP.

All the reviewers appreciated the authors’ willingness to improve and revise the AOP in the upcoming weeks following the end of review TC.

There was consensus among the reviewers on the issues raised from the reviewing process. Once the changes proposed to address these issues are implemented, the reviewers would support that this AOP gets submitted for approval and be subsequently published. The authors are expected to concretise actions arising from the review and the teleconference before April 2022.

4. Summary of planned revisions

An extensive revision of the AOP was required and agreed by the two authors present at the TC (see also section 3).

Authors were asked to address reviewers' point by point comments. These answers were timely and extensively done by the authors. The responses to comments are accessible in Annex 2, sections 1, 2 and 3 respectively for Reviewer 1, Reviewer 2 and Reviewer 3.

All reviewers' comments and suggestions have been taken into consideration. Either the authors have already changed the text on the AOP-Wiki at the time the report is submitted (April 2022) or are going to complete the changes.

The main points addressed are listed below:

1. Scientific evidence

An update of the literature cited in several places was required. The authors defined a series of complex queries on developmental vascular toxicity (DVT) to mine the literature with 'AbstractSifter' tool on November 30, 2021. This returned 169 PubMed records, of which ***93*** were new.

2. Overview of the AOP

AOP description, graphical representation including KERs (agreed but still to be done at the time the report is submitted – April 2022).

MIE:VEGFR2, inhibition

> One of the main comments was that some KE could be upstream of the MIE305. This was acknowledged in previous versions of the AOP. However, it was dismissed following internal review. The 'Key Events Component' of MIE:305 explicitly states decreased "vascular endothelial growth factor receptor 2 binding" as the underlying premise for a quantitative effect on the VEGF system. The description of MIE:305 states that "… decreased VEGFR2 binding is the quantitative basis for an effect of stressors on VEGFR2 activation of the 'master switch' in developmental angiogenesis." MIE description has been revised (see page 25).
>
> The section "How MIE is measured or detected" has been revised too.

KEs (all updates completed):

> - KE 28: Reduction of angiogenesis: a lack of references was noted and revision has been done (see box 5). The section "how KE28 is measured or detected" was revised (box 6).
>
> - KE 110: Impairment of endothelial network. The domain of applicability has been revised (see box 7). The KE description and the section "how it is measured or detected" have been updated with recent supportive literature (see respectively box 8 and box 9).

- KE 298: insufficiency-vascular. One of the main comments was that consequences rather than a description was given. The description and section on how measuring and detecting have been completely re-written and are accessible boxes 10 and 11.

AO 1001-Developmental defects, increase - Revision was done on this section (see box 16)

KER - Updates were suggested for most of the KER description.

The following comments have been taken into account by the authors: revised description and supporting evidence for KER 335 (Inhibition, VegfR2 leads to Reduction, Angiogenesis) - accessible boxes 12 and 13; revised KER 36 description (Reduction, Angiogenesis leads to Impairment, Endothelial network) - box 14; KER 125 (Impairment, Endothelial network leads to Insufficiency, Vascular) has been revised (see box 17) and finally KER1036 (Insufficiency, Vascular leads to Increased, Developmental Defects) extensive revision is accessible box 18.

3. Weight of evidence

The DoA aspects have now been more precisely addressed in the revised DoA overall (see box 1) and Essentiality (see box 2) further described to explicitly point to endothelial tip cell sprouting including the citations to Belair et al. (2016) and related studies.

The WoE sections have been extensively revised and updated (see the various boxes holding the proposed text for all revised sections of AOP43). At the time the report is submitted – April 2022 – the authors have not yet had time to complete the summarising table suggested (by Reviewer3) but will do so.

All modifications are accessible in Annex 2 of this report.

The extensive revision was timely done and implemented in the AOP-Wiki on January 22nd 2022. All changes have been reviewed by the panel.

Further discussion

December 2021

Before proceeding to drafting the rebuttals, for those comments with which the authors did not agree, a short TC (15 min) was organised at the initiative of Tom Knudsen with the review manager, to discuss specific issues about changes to be incorporated into the AOP (December 20th 2021).

All answers were sent by December 24th to the reviewer manager.

January 2022

Except from the graphical representation and the WoE summarising table, all reviewers' comments were taken into account. The panel and review organiser agreed that the authors did a very extensive revision of the AOP and that it is now suitable to be submitted to the EAGMST for approval.

5. Outcome of the scientific review

Following initial review, all the reviewers of this AOP felt that the authors had to improve the AOP. The reviewers though acknowledged the significant contribution of the authors through the development of this AOP to the AOP-Knowledgebase. The reviewers also acknowledged the amount of work that has gone into other AOPs sharing some Key Events with AOP43 and that, together, will build a comprehensive AOP network.

The reviewers devoted significant amount of their time to provide constructive comments, editorial changes and additional literature. All these materials have been made available to the authors. There was consensus among the reviewers on the issues raised from the reviewing process. The authors replied to the different comments and agreed to implement most of the suggested changes by updating and making changes to specific sections of this AOP.

All the reviewers appreciated the authors' willingness to improve and revise the AOP in the upcoming weeks following the end of review TC and felt that once the AOP has been modified according to the reviewers' recommendations it would be appropriate that this AOP is submitted for OECD approval and subsequently published. The authors were expected to concretise actions arising from the reviews and the teleconference.

In January 2022, the panel also reviewed the written responses provided by the authors and available in Annex 2. They agreed that the authors significantly improved the descriptions of the proposed AOP 43 and fully addressed or discussed all major issues raised in the comments. Except from the graphical representation and the WoE summarising table, all reviewers' comments were taken into account. The panel and review organiser agreed that, pending completion of remaining revisions, the AOP is now suitable to be submitted to the EAGMST for approval.

Annex 1: Reviewers' panel

Name	*Affiliation*	*Expertise called for the review*
Dr Makiko Kugawata	Division of Cellular and Molecular Toxicology, CBSR, NIHS, Japan	Angiogenesis
Dr Maria Bastaki	European Food Safety Agency (EFSA),	Endocrine disrupter chemical hazard, angiogenesis, vascularisation
Dr Nils Ohnesorge	German Federal Institute for Risk Assessment (BfR)	Neuronal development, drug screening for anti angiogenic, metabolism, blood flow and endothelial cell, zebrafish model

Annex 2: Authors reply to reviewers' comments (24th December 2021)

AOP authors' written response on reviewers' comments are provided in sections 1-4 below of Annex 2.

December 24, 2021

FROM: Tom Knudsen

TO: Jean-Baptiste Fini, external review manager

CC: Nils Ohnesorge, M Kuwagata, Maria Bastaki, Nathalie Delrue, Nancy Baker

RE: Response to external review of Aop43, **"**Disruption of VEGFR Signaling Leading to Developmental Defects" [https://aopwiki.org/aops/43]

****

Dear all,
Thank you for coordinating the external review of Aop43 and compiling the comments from three independent expert reviewers, conducted in summer 2021. This document summarizes our response to each comment, which was drawn from a PDF snapshot provided by the OECD Secretariat [https://aopwiki.org/aopwiki/snapshot/pdf_file/43-2021-02-23T17:25:31+00:00.pdf]. We reviewed the "*Draft Guidance Document for the scientific review of Adverse Outcome Pathways*" approved 22 July 2020 by the OECD Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) and are keenly aware of the complexities in both constructing and evaluating an Aop43 for OECD endorsement. Please note that we have a peer-reviewed publication on 'Systems Modeling of Developmental Vascular Toxicity' [Saili et al. 2019, PMID:32030360] that delves into the biology and toxicology of this AOP under the general context of 'developmental vascular toxicity', which is the short title of Aop43. We will refer to this concept by the acronym 'DVT' in responding to the general and specific comments from the summer 2021 external review.

Annex 2 - Section 0 - GENERAL COMMENTS (GC):

GC.1: *All reviewers agree with the fact that this AOP needs more work before being endorsed. All reviewers agree with the fact that literature is not up to date. The three reviewers ask for an update. Please consider replying to all reviewers and incorporate the suggested literature.*

Response: To address the general comment, we defined a series of complex queries on DVT to mine the literature with our 'AbstractSifter' tool on November 30, 2021. This returned 169 PubMed records, of which ***76 were cited previously and 93 are new*** to this response.

GC.2: *Does the scientific content of the AOP reflect current scientific knowledge on this specific topic - in general this point is satisfying. An update on stressors (R3) and literature (R1) is suggested.*

Response: Our updated literature includes 57 references for chemical stressors in 33 targeted studies and 24 high-throughput screening (HTS) publications. In addition, several papers addressing growth factor and/or genetic manipulation are among the 14 citations that support the MIE for Aop43 (MIE:305). Details provided under responses to specific reviewer comments.

GC.3: *Weight of evidence is not appropriate according to the reviewers because there is not enough justification. Even though events are mostly well described they agree that the vast majority of the KE and KER are correctly weighted, justifications are not present and some precisions are needed.*

Response: We were at least pleased to see that the reviewers found KEs and KERs to be *"... mostly well described ...*" and "*... correctly weighted ...*". Some of that justification is referenced in the optional 'Background' section. To address the comment, we provide more precise justification in the appropriate sections of revised Aop43 with specific references, where appropriate.

GC.4: *The available data in the literature needs to be presented in a structured way, demonstrating quantitative relationships of MIE leading to AO. Most KEs and KERs had essential sections missing completely or not used in alignment with the Guidance Document. (see specific comment form R1)*

Response: We reviewed the "?" icon for each section in Aop43 to assess required information beyond optional information provided. Our AbstractSifter supporting Aop43 was valuable in structuring the landscape of literature supporting the various KEs and KERs. We will also confirm alignment with the "*Users' Handbook supplement to the Guidance Document for Developing and Assessing Adverse Outcome Pathways*" (OECD 2018, http://dx.doi.org/10.1787/5jlv1m9d1g32-en).

Annex 2 – Section 1 - Reviewer #1

***R1.1* Does the AOP incorporate all appropriate scientific literature and evidence?** *I think it needs more references to support the content presented. A few examples are below. In the pVDC signatures, references are lacking for involvement of VCAM-1, cytokines and chemokines, angiogenic growth factors, angiogenic sprouting, molecular players and pathways for vascular stabilisation. The reference for Knudsen and Kleinstreuer 2011 and Kleinstreuer et al., 2014 are used repeatedly but perhaps more specific references would be better.*

Response: ***Thank you for these comments.*** Importantly, DVT is 'embryogenic' and covers novel processes such as vasculogenesis (*de novo* blood vessel formation), angiogenesis (new vessels sprouting from existing networks), and vascular remodeling (stabilization, patterning) that may differentiate the developmental pathways from other pathogenetic domains such as cancer, immunology, and cardiovascular disease. We have published extensively on AOP-based DVT since 2011: (i) *Disruption of embryonic vascular development in predictive toxicology* [Knudsen and Kleinstreuer, 2011]; (ii) *A computational model predicting disruption of blood vessel development* [Kleinstreuer et al. 2013]; (iii) *Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms* [Kleinstreuer et al. 2014]; (iv) *Immediate and long-term consequences of vascular toxicity during zebrafish development* [Tal et al. 2014]; (v) *Screening for angiogenic inhibitors in zebrafish to evaluate a predictive model for developmental vascular toxicity* [Tal et al. 2017]; (vi) *Embryonic vascular disruption adverse outcomes: Linking high throughput signaling signatures with functional consequences* [Ellis-Hutchings et al. 2017]; (vii) *Systems modeling of developmental vascular toxicity* [Saili et al. 2019]; and (viii) *A cross-platform approach to characterize and screen potential neurovascular unit toxicants* [Zurlinden et al. 2021]. We recognize and appreciate the need to render connections to other potential AOPs and employed controlled vocabularies to the best of our understanding in citing specific references amongst 76 cited from the previous Aop43 and 93 new cites in revised Aop43.

***R1.2* Overview of AOP:** *More specific references would be good to provide here, e.g. "Genetic studies have shown that perturbing these signals can lead to varying degrees of adverse consequences, ranging from congenital angiodysplasia to fetal malformations and embryolethality" and other statements.*

DoA response summary: Our literature search identified 42 publications focused on direct linkages between adverse developmental outcomes and disruption of blood vessel development. Some were mechanistic studies on blood vessel morphogenesis as the primary pathway. Others were genetic or pharmacological models of human pregnancy and development. The description was rewritten and referenced (see box 1) to improve alignment with the main points on the 'Biological Domain' (box 1):

- Biological context: *vascular toxicity*
- Taxa: *zebrafish, mouse, rat, human*
- Life-stage: *embryonic (organogenesis)*
- Sex: *unspecific*

1. Domain of Applicability (DoA revised)

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.

R1.3 Essentiality of KE:

Comment: *Belair et al. 2016 is the reference that directly supports that the endothelial cell tip is the critical VEGFR2 responsive cell type specifically and should be cited in the respective statement.*

Response: This is cited (see box 2).

Comment: *What is the reference for pluripotent stem cells and aortic explants? The Sarkanen et al 2010 paper reports on tubule network formation in HUVEC + fibroblast co-cultures, the Kleinstreuer et al, 2013 reports on a predictive in silico model, and the Tal et al. 2014 on zebrafish.*

Response: References are included for Belair et al. 2015; Sinha and Santoro, 2018; Li et al. 2018; Galaris et al. 2021 (see box 2).

Comment: *Arsenic is non-specific and probably not a good example (Shirinifard et al. 2013 - is this the right reference?).*

Response: it is the correct reference, although the effective concentrations shown in that study are well-above environmentally realistic exposures.

Comment: *In vivo essentiality in embryo development or female fertility, uterine cycle and placentation are not supported with references, neither are the rodent whole embryo culture and transgenic endothelial zebrafish reporter lines.*

Response: A new sentence was inserted: "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]" (see box above).

Rebuttal: We disagree with the comment about rodent whole embryo culture and zebrafish lines. The AOP guidance states the essentiality of Key Events aims to *"... summarise briefly the nature and numbers of investigations in which the essentiality of KEs has been experimentally explored either directly or indirectly*". While an apical *in vivo* outcome of regulatory value is certainly the goal of any AOP, the modular data from *in vitro* and reporter zebrafish models are important inclusions for evidentiary support.

***Comment**: Human link between in utero vascular disruption and limb etc defects: Husain et al. 2008 is descriptive of epidemiology related to birth defects but does it give enough evidence to support the link with vascular disruption? Gold et al. 2011 suggests a vascular association but is by no means evidence of causation.*

Response: Agreed. We revised this assertion to indicate a correlation that "… 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]." Please note that this comment and response is now related to the 'Domain of Applicability' (box 1).

Essentiality Response Summary: The paragraph in box 2 was rewritten and referenced to improve alignment with the main points on the 'Essentiality of the Key Events' from the guidance document:

- Pathway: *Vascular Endothelial Growth Factor (VEGF) signaling system*
- Master gene: *VEGFR2 (alias KDR/Flk-1) receptor tyrosine kinase*
- Upstream: *VEGFR2 expression, liganding, kinase activation*
- Downstream: *sprouting, migration, adhesion, proliferation, apoptosis*
- Microarchitecture: *network formation, lumenization, stabilization, remodeling*

2. Essentiality of the Key Event (revised)

The Vascular Endothelial Growth Factor (VEGF) pathway is a critical regulatory system for assembly of embryonic blood 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),which proliferate in the wake of an angiogenic sprout; macrophage/microglial cells (MCs), which release cytokines, chemokines, and growth factors; and stromal cells (SCs) that 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].

R1.4 WOE for MIE and AO

Comment: *I would agree it is strong. Supporting references: Belair et al. 2016 VEGF and vasculature in vitro and eye in vivo; Nguyen et al. 2017 in vitro; Tal et al. 2017 zebrafish embryo; McCollum et al. 2017 zebrafish embryo; Ellis-Hutchings et al. 2017 in rat whole embryo culture (WEC) assay and zebrafish embryo but not specific to VEGFR; Saili et al. 2019 review of the WoE for this AOP and seems to have more complete reference list; Zurlinden et al. 2020 neurovascular development.*

Response: We concur that zebrafish embryo and rat WEC platforms do not directly speak to the specificity of the MIE:305 in VEGFR2 disruption. There are distinct advantages to point out for each system in rendering a useful AOP for mechanistic and regulatory toxicology.

The zebrafish fluorescence reporter is driven by KDR gene regulatory elements. Changes to the developmental pattern infers a molecular response specific to VEGFR2-positive endothelial cells. As such, it is an indicator of alterations to blood vessel patterning. Although not directly linked to an inhibition of VEGFR2 binding (MIE:305), the platform was amenable to screening large numbers of environmental chemicals in ToxCast [Tal et al. 2017; McCollum et al. 2017]. ToxCast has *in vitro* profiling data on 1065 chemical compounds across over 1100 diverse biological assays, many of which can be related to the angiogenesis cycle in general and the VEGFRs in particular [Knudsen and Kleinstreuer, 2011; Kleinstreuer et al. 2013; Saili et al. 2019]. This provides an opportunity to place stressor-MIE interactions for VEGFR2 in the context of molecular mechanisms for blood vessel formation across different vascular beds within the embryo [reviewed by Eberlein et al. 2021].

The rat WEC work uniquely correlated the effect of two ToxCast angiogenesis inhibitors (TNP470, a synthetic fumigillan analog; and 5HPP-33, a synthetic thalidomide analogue) across zebrafish and mammalian systems tuned for predicting human developmental toxicity [Ellis-Hutchings et al. 2017]. The extent which either compound interferes with VEGFR2 binding is not known for sure; however, in [Saili et al. 2019] we used RNAseq profiling to assess how the embryonic transcriptome reacts to TNP470 and 5HPP-33 as their concentrations are increased from no observable effect to embryotoxicity, which is linked to intermediate steps on the angiogenesis cycle during embryotoxicity. Again, we realize this information may not be specific to the inhibition of VEGFR2 binding. Indeed, the RNAseq profile identified molecular signaling pathways that are not currently incorporated into Aop43. But the study also demonstrated the value of Aop43 for assembling quantitative information on functional vascular development to make use of an AOP for assessing WoE and guiding the exploration of KERs.

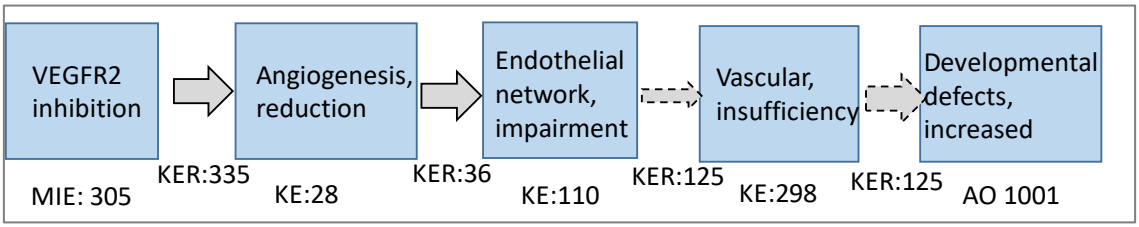
R1.5 MIE

Comment: For VEGFR2. *The supporting literature provided is rather old – PubMed results in ca 2700 articles, of which >260 reviews in the last 5 years. VEGFR and develop* (in Tit/Abst): 1746 results since 1995; 762 since 2014; 274 since 2019. VEGFR and develop* (in Tit/Abst) Reviews: 482 since 1995; 206 since 2014; 68 since 2019. VEGFR and developmental (in Tit/Abst): 101 results since 1999; 39 since 2014; 16 since 2019. VEGFR and developmental (in Tit/Abst) Reviews: 20 since 2001; 9 since 2014; 0 since 2019 (11 between 2012-2018).*

Response: The reviewer has pointed out the large number of publications and review articles on VEGFR and development in PubMed. VEGFR2 belongs to Class IV superfamily of transmembrane receptor tyrosine kinases (RTKs) that play critical roles in the development and progression of many types of cancer, resulting in an extensive literature base on molecular properties, cellular effects, and pharmacology/toxicology.

Our literature search with our PubMed AbstractSifter tool [Baker et al. 2017] was most recently updated for DVT on November 30, 2021. We started with a broad search strategy to run multiple (29) complex queries based on terms and complex queries mined in the PubMed for article title, abstract text, and Medical Subject Headings (MeSH). This returned 22,785 results. After filtering-out redundant records, the corpus of articles relevant to embryo-fetal development was trimmed using automated search of terms such as VEGF, vasculogenesis, angiogenesis, toxicity, assays, high-throughput screening, and so forth. This was followed by manual curation for vasculogenesis-angiogenesis and/or adverse developmental outcomes. The resulting DVT corpus included 169 PubMed records, of which **76 were cited previously and 93 are new to this response**.

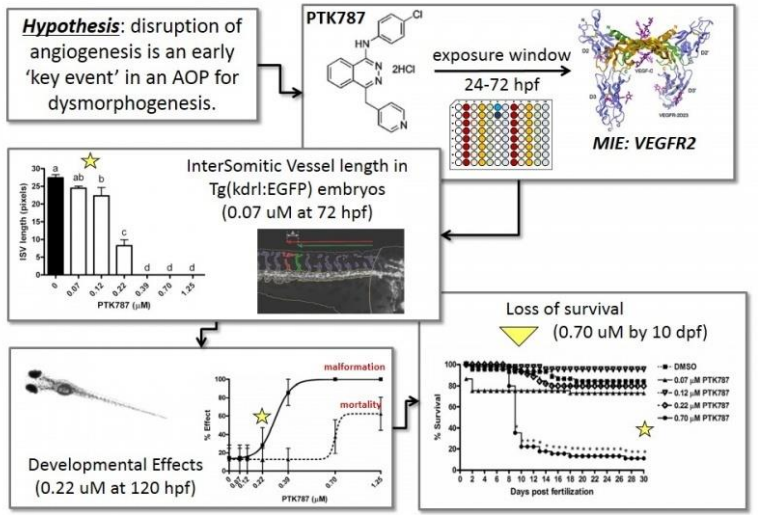
As a general organizing principle, we mapped these PubMed records to specific Event IDs assigned to Aop43 in the sequence below. Literature support for VEGF-signaling in developmental angiogenesis has 68 results since 1995; 28 since 2014; and 12 since 2019. Again, these numbers are smaller than the reviewer's simple search queries most likely because of our winnowing to DVT. Many of these articles would of course support WOE for more than one element in the sequence below, but for simplicity we assigned only one Event ID – the most appropriate, to each article. The distribution of articles across the sequence is shown in the table below. At least 24 papers support evidence for the point of interaction between stressor (inhibition of VEGFR2) and angiogenesis (KE:28).



Sequence	Type	Event ID	Title / Short name	Approx. # references cited (overlapping)
1	MIE	305	Inhibition, VEGFR2	17
2	KER	335		25
3	KE	28	Reduction, Angiogenesis	28
4	KER	36		25
5	KE	110	Impairment, Endothelial network	76
6	KER	125		38
7	KE	298	Insufficiency, Vascular	58
8	KER	1036		17
9	AO	1001	Increased, Developmental Defects	14
1 to 9	AOP	Aop43	review articles	16
0	stressor	reference	angiogenesis inhibitors	33

0	stressor	HTS	high-throughput screen	24
TOTAL				**169**

File:KleinstreuerKnudsenAOPVascularDisruption.jpg > File:AOP ZFE.jpg



Data supporting the quantitative role of MIE:305 was shown for Vatalanib (PTK787), published in a zebrafish developmental toxicity study evaluating concentration-responses. That study, previously labelled as 'in press', was published [Tal et al. 2014]. We will update this image file.

***Comment*: For Vatalanib.** *Gustafsdottir et al. 2008 (6 different inhibitors) (to check); Wood et al. 2000 IC50 for Vatalanib; 2002; Kendall et al. 1999 (to check).*

Response: We rechecked all three studies, originally cited in Aop43. Gustafsdottir et al. (2008) evaluated 6 mechanistically diverse inhibitors on VEGF-A_{165} activation of VEGFR2 in solubilized cell extracts: (i) commercial VEGF-A aptamer (t22-OMe); (ii) 2'-fluoropyrimidine RNA-based aptamer to VEGF-A_{165}; (iii) recombinant competitive protein (PGF); (iv) neutralizing VEGF-A monoclonal antibody; (v) neutralizing VEGFR2 monoclonal antibody C27; and (vi) a synthetic GFA-116 low molecular weight inhibitor of VEGF-A binding to VEGFR2. That study did not use Vatalanib but does provide definitive evidence that MIE:305 can be engaged either by blocking ligand binding or subsequent kinase activation. Wood et al. (2000) reported that Vatalanib (PTK787) is a potent pharmacological inhibitor of VEGFR2 kinase activation. They looked at its effects on other angiogenesis RTKs and found higher concentrations could inhibit PDGFRβ. The evidence for Vatalanib's selectivity followed the rank order VEGFR2 > PDGFRβ >>> EGFR, FGFR1. Kendall et al. (1999) reported inhibition of KDR-kinase (VEGFR2) activity in cell extracts with Indolinone. Although an angiogenesis inhibitor, unlike Vatalanib Indolinone is a multikinase inhibitor and thus is not selective for VEGFR2.

Synergistic inhibition across several potential MIEs (VEGFR-2, PDGFRβ, FGFR-1) is a strong qualifier for clinical efficacy, but less so for supporting the specificity of MIE:305. Anlotinib, for example, is a more potent but less specific inhibitor of angiogenesis than Sunitinib acting on VEGFR-2, PDGFRβ, and FGFR1 pathways [Lin et al. 2018]. A fungal metabolite, epoxyquinol B (EPQB) is anti-angiogenic through inhibition of even broader pathways (VEGFR2, PDGFR, EGFR, FGFR) [Kamiyama et al. 2008].

Comment: *References for pharmacological compounds are missing. What is the range of their specificity for VEGFR?*

Response: Two pharma compounds in 'Chemical Table' currently support MIE:305: Vatalanib (PTK787) and Sunitinib (indolinine derivative). These potent anti-angiogenic antagonists inhibit VEGFR2 kinase activity upon activation of the receptor; however, at higher concentrations they also inhibit PDGFRβ kinase activation [Wood et al. 2000]. Uploading the relevant information to the 'edit text' of Aop43 would enable AOP-Wiki's automatically generated 'Event Evidence' to fill in fields currently missing from the 'AOP Evidence' section. As to the range of specificity, Belair et al. (2016b) evaluated 9 mechanistically diverse anti-angiogenic drugs in a human endothelial sprouting assay. The point-of-departure effect on anti-angiogenic potency followed the rank order: Vatalanib (10 nM) > Sunitinib malate (20 nM) > Combretastatin A4 (100 nM) > Temsirolimus (0.2 uM) > SB-3CT (0.5 uM) > Withaferin A (0.8 uM) > Thalidomide (2 uM), SU5416 (2 uM) > Nilotinib (7 uM). Therefore, **Vatalanib represents the strongest stressor for MIE:305** followed in turn by Sunitinib and other mechanistically diverse compounds that are less specific or sensitive inhibitors of VEGFR2 activation.

Comment: *Tal et al. 2014: only this one is provided to support the MIE. How specific is the MOA for vatalanib? Example of more inhibition of FGF-induced angiogenesis: Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis in vivo and in vitro. Tille JC, Wood J, Mandriota SJ, Schnell C, Ferrari S, Mestan J, Zhu Z, Witte L, Pepper MS. J Pharmacol Exp Ther. 2001 Dec;299(3):1073-85.*

Response: The main MIE qualifier for AOP elucidation is a quantitative relationship between mechanistic interaction with stressor (MIE) and apical effect (AO). Possible crosstalk between endogenous angiogenic pathways (eg, VEGF, FGF) complicates this specificity [Tille et al. 2001]. Indeed, 58 distinct RTKs occur in 10 classes grouped by ligand specificity, cell-type, and downstream consequences. Several classes have angiogenic roles, including Class IV (VEGFRs), Class III (PDGFRs), Class I (EGFRs) and Class 5 (FGFRs). Multi-class synergy may be useful clinically for targeting angiogenesis as a mode-of-action (MOA); however, any pharmacological inhibitor, no matter how specific, is not in itself sufficient justification for MIE support.

With its focus on Vatalanib, Tal et al. (2014) remains the most relevant study for demonstrating a holistic relationship between MIE:305 stressor and DVT. And yet as a zebrafish developmental study exposed during organogenesis Tal et al. (2014) has two limitations as a robust Aop43 qualifier: (i) did not measure VEGFR2 biochemical activity following exposure; and (ii) did not evaluate apical effects on pregnancy (for obvious reasons). The first limitation is satisfied by strong evidence supporting a quantitative relationship between Vatalanib and VEGFR2 inhibition, noted above. The second limitation is reconciled by direct evidence for angiogenesis-related development effects upon genetic disruption of VEGFR2 function in zebrafish [Chan et al. 2002; Eberlein et al. 2021] as well as mouse [Fong et al. 1995; Shalaby et al. 1995; Ferrara et al. 1996; Carmeliet et al. 1996; Abbott and Buckalew, 2000; van den Akker et al. 2007]. This information is noted in the revised 'Domain of Applicability' (see box 1).

Comment: *Reference to support the high evidence for mouse DoA (given for VEGF-A mutant mice: Ferrara et al. 1996; Carmellet et al. 1996 and one reference for the receptor mutant mice: Shalaby et al. 1995), or for human evidence is not provided. Reference is made only for zebrafish.*

Response: These studies were referenced. Functional inactivation of VEGF-signaling in mouse 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]. Since VEGFR1 can be activated by other growth factors (e.g., Placental Growth Factor), the specificity for VEGF signaling was assessed by targeting the *Vegf* gene. These studies showed impaired angiogenesis and hematopoeisis in *Vegf(+/-)* heterozygotes leading to midgestational embryolethality [Ferrara et al. 1996] and even stronger effects deficiencies in *Vegf(-/-)* homozygotes [Carmeliet et al. 1996]. The progressive severity of effects for VEGF-signaling was gene dose dependent, indicating a quantitative gene dose-dependent effect of loss of VEGF binding.

Additional citations are provided in the DoA (Domain of applicability) section of the AOP overview as evidence for dysmorphogenesis induced by anti-angiogenic compounds 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] and human malformations correlated with genetic or environmental factors that disrupt vascular development [Husain et al. 2008; Gold et al. 2011; Vargesson and Hootnick, 2017]. All are cited under appropriate KE sections.

Comment: *For the MIE, the statement "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" suggests that each of these is a separate MIE subject to chemicals with very different structures… However, the fact that disruption of VEGFR2 has different origins is not unique to this receptor… Any receptor disruption does (e.g. ligand availability, reduced expression, etc).*

Response: Fully agree! Our earlier versions of Aop43 argued that some KE can be upstream of the MIE; however, the internal reviewers disagreed. So rather than building a separate AOP for each origin of VEGFR2 inhibition Aop43 looks at this as the 'master switch' in developmental angiogenesis. The 'Key Events Component' of MIE:305 explicitly states decreased "vascular endothelial growth factor receptor 2 binding" as the underlying premise for a quantitative effect on the VEGF system. The description of MIE:305 states that "… decreased VEGFR2 binding is the quantitative basis for an effect of stressors on VEGFR2 activation of the 'master switch' in developmental angiogenesis." (see concluding statement in the revised MIE box 3, shown below).

Comment: *Reference for the evidence that ECM breakdown releases VEGF-A from VEGFR1?*

Response: The statement was motivated by *Rosen and Lemjabbar-Alaoui (2010) Sulf-2: An extracellular modulator of cell signaling and a cancer target candidate. Expert Opin Ther Targets 14: 935–949*. The observation was that sulfatases released during tissue injury resulted in proteoglycan breakdown and de-sequestration of VEGF-A; however, given the aforementioned de-emphasis on mechanisms of VEGF-A release upstream to VEGFR2 liganding, the statement was removed from the MIE description.

MIE Description Response Summary: Box 3 shows the revised MIE description based on the comments/responses noted above. Key points for MIE:305 and KER:335 are as follows:

- Specialized KE: *VEGFR receptor activation*
- Stressor: *genetic (physiological inactivation), environmental (drug/chemical)*
- Point of interaction: *VEGF binding to VEGFR2*
- Biological system: *blood islands, angiogioblasts, endothelium*

3. MIE Description (revised)

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.

R1.6 How it is Measured or Detected (p 8)

Comment: *Assays for each preceding event that leads to reduced VEGFR2 activity. For receptor binding assay Gustafsdottir et al. 2008. For receptor capacity (density, expression levels) and bioactivity: a couple of references are given – and 2 ToxCast assays for each. VEGFR2 capacity assay based on protein levels? Kleinstreuer et al. 2014 – other? Bioactivity Knudsen et al. 2009; Sipes et al. 2011 other?*

Response: A number of targeted and high-throughput assays are used to quantitatively 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. The ToxCast assay portfolio is accessible from the EPA Computational Toxicology Chemicals Dashboard (https://comptox.epa.gov/dashboard/). You can search assays by gene name. There are 6 biochemical (cell-free) assay features for human VEGFR1, VEGFR2 and VEGFR3 under the

'NovaScreen' (ToxCast_NVS) platform. Chemical effects data from assay component NVS_ENZ_hVEGFR2 was analyzed relative to DMSO as a neutral control and Staurosporine as a positive control [Knudsen et al. 2011; Sipes et al. 2013]. *Please note the dates of these references were incorrectly written in the previous draft.* A multiplex assay described under the 'BioSeek' (ToxCast_BSK) platform exists for VEGFR2. It measures increased or decreased VEGFR2 immunoreactive protein by ELISA in primary human umbilical vein cells (HUVEC) conditioned to simulate proinflammation. A change in VEGFR2 receptor density infers endothelial capacity for $VEGF_{165}$ binding [Kleinstreuer et al. 2014]. These details have been updated more precisely in the revised Aop43 (see box 4).

Comment: *Gene expression with standard array assays and targeted non-array methods: Dumont et al. 1995; Abbott et al. 2000; Drake et al. 2007; Murakami et al. 2011, The models giving phenotypic evidence are not measuring the MIE but they are downstream KE… and don't belong under MIE section (Dumont et al. 1995; Abbott et al. 2000; Drake et al. 2007; Murakami et al. 2011) (p.9).*

Response: This information (updated) has been relocated to KE sections where appropriate to for more general descriptions of angiogenesis versus information specific to the embryo. We agree that it provides a more appropriate workflow for lucid organization.

MIE detection Response Summary: Box 4 shows the revised MIE description based on the comments/responses noted above. Key points for MIE:305 and KER:335 are as follows:

- Specialized KE: *VEGFR2 receptor activation*
- Stressor: *genetic (physiological inactivation), environmental (drug/chemical)*
- Point of interaction: *VEGF binding to VEGFR2*
- Biological system: *blood islands, angiogioblasts, endothelium*

4. How MIE is Measure or Detected (revised)
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 nearly 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 upon 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].

R1.7 KE:28 Reduction of angiogenesis
***Comment**: DoA, Sprouting EC: Belair et al. 2016 is a very strong reference. TG zebrafish embryos: Tal et al. 2016 (DoA) applicability of model to human based on key nodes across species (only one reference?)*

Response: Endothelial tip cell sprouting is the critical element for KER:335, linking MIE:305 with KE:28. The DoA aspects have now been more precisely addressed in the revised DoA overall (see box 1) and Essentiality (see box 2) descriptions to explicitly point to endothelial tip cell sprouting including the citations to Belair et al. (2016) and related studies. These revised descriptions reference the applicability of the model to humans based on key nodes across species is now referenced for zebrafish.

Overall, 24 references support zebrafish goals for: (i) mechanistic evaluation of vascular development [Chan et al. 2002; Jin et al. 2005; Gerri et al. 2017; Jin et al. 2017; Sinha et al. 2018; Eberlein et al. 2021]; (ii) quantitative screening of anti-angiogenic compounds [Tran et al. 2007; Yozzo et al. 2015; Beedie et al. 2016b; Ellis-Hutchings et al. 2017; McCollum et al. 2017; Tal et al. 2017]; and (iii) targeted evaluation of specific compounds [Jang et al. 2009; Jung et al. 2009; Bonventre et al. 2011; Mahony et al. 2013; Shirinifard et al. 2013; Beedie et al. 2015; Beedie et al. 2016b; Beedie et al. 2017; Kotini et al. 2020]. Several of these focus on thalidomide as a human teratogen and disrupter of developmental vasculogenesis. Thalidomide and its analogs in particular have been screened and investigated for shared teratogenicity on vascular development

in the zebrafish embryo and chick chorioallantoic membrane [Mahony et al. 2013; Beedie et al. 2016a,b; Beedie et al. 2017]. This may be most relevant for KE:28 and details now cited in its revised description (see box 5 below), and cited elsewhere as may be appropriate.

Comment: *KE description: Only one reference for a complex process? Knudsen and Kleinstreuer, 2011.*

Response: As the Reviewer points out developmental angiogenesis is a complex process. Our previous reviews [Knudsen and Kleinstreuer, 2011; Saili et al. 2019] were comprehensive in referencing the literature supporting an early version of Aop43, which was not specific to the VEGF system. Much of that background covered HTS-based mapping different assays to the overall AOP and has now gotten tangled during refinement of Aop43 following two internal reviews. With this revision, relevant descriptions for 'blood vessel morphogenesis' have been parsed to KE:28 and other nodes as appropriate. The need for more precise and updated referencing for KE:28 is duly noted, and details have been updated with original referencing.

'Blood vessel development' in Gene Ontology (GO) maps to GO term GO:0001568, defined as "*The process whose specific outcome is the progression of a blood vessel over time, from its formation to the mature structure. The blood vessel is the vasculature carrying blood"*. The most applicable subordinate for Aop43 is 'Blood Vessel Morphogenesis' that maps to 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*"). There are at present 660 genes manually curated to this process in The Mouse Gene Ontology Browser (http://www.informatics.jax.org/vocab/gene_ontology/) from MGI This is the immediate parent to the following list of GO annotations (shown with the number of curated gene associations in the mouse phenotype browser):

blood vessel morphogenesis (660 genes)
- angioblast cell migration (1 gene)
- **angiogenesis (545 genes)**
- apoptotic process involved in blood vessel morphogenesis (0 genes)
- artery morphogenesis (81 genes)
- blood vessel lumenization (2 genes)
- coronary vasculature morphogenesis (32 genes)
- glomerulus vasculature morphogenesis (4 genes)
- **negative regulation of blood vessel morphogenesis (110 genes)**
- retinal blood vessel morphogenesis (5 genes)
- selective angioblast sprouting (0 genes)
- **vasculogenesis (96 genes)**
- venous blood morphogenesis (12 genes)

Three annotations shown in boldface account for 593 (89.8%) of the 660 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*"). Gene numbers curated

to blood vessel morphogenesis (BVM) with overlapping for GO subordinates (593) looks like this:

BVM	*vasculogenesis*	*angiogenesis*	*(-) regulation*
660	96	545	110
7	7	7	7
48	48	0	0
41	41	41	0
393	0	393	0
103	0	103	103
67	0	0	0

Of these curated gene associations, 48 (7.2%) were specific to vasculogenesis, 393 (59.5%) to angiogenesis, and 48 (7.2%) in common to both processes. Overall, the genes annotated for the VEGF system had 7 genes: *Flt1 (Vegfr1)* and its ligand (*Vegfb*)*; Kdr (Vegfr2)* and its ligand (*Vegfa*)*; Flt4 (Vegfr3)* and its ligand (*Vegfc*). The other, *Vegfd*, codes for a ligand active on either VEGFR2 or VEGFR3. *Vegfr2* alone mapped to both vasculogenesis and angiogenesis consistent with its critical pro-angiogenic role, while *Vegfr1* alone mapped to negative regulation of BVM consistent with its role as an endogenous angiogenesis inhibitor. Although the genetic signals and responses for vasculogenesis and angiogenesis may differ, disruption of VEGFR2 is common to both. Since disruption of vasculogenesis would ultimately lead to a reduction in angiogenesis, both processes are in-scope for KE:28. The critical effect on developmental angiogenesis is, therefore, best described in the GO term for 'negative regulation of blood vessel morphogenesis'. These details are included with updated citations in the revised description of KE:28 (see box 5 below).

KE:28 Response Summary: Box 5 shows the revised KE:28 description and Box 6 the revised section on relevance and reliability of the methods with which KE:28 can be measured. Key points are as follows:

- Stressors: *anti-angiogenic compounds (drugs/chemicals)*
- Taxonomic Applicability: *zebrafish, chick, mouse, rat, human*
- Lifestages: *embryonic (organogenesis)*
- Sex Applicability: *unspecific*

5. Key Event Description (KE:28 revised)

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 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.

Comment: *How is it measured: again single references only*

Response: Diverse assays are used to detect or measure the biological states represented in KE:28 (see box 6 below for revised text). Broadly stated, the methods 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. The original description was largely focused on our own interests in HTS platforms for predictive toxicology. The revised version (box 6 below) is more broadly driven by the scientific challenge to elucidate

pro-angiogenic pathways and motivated by management of neovascularization for disease progression, drug discovery, and assessing environmental chemicals.

While it is not possible to cover the breadth of this field in KE:28, key assays are cited with short descriptions of scientific confidence in the measurement approach specifically as it is fit for purpose of assessing drug and chemical effects on developmental angiogenesis. Some are well-established commercial platforms using molecular probes and antibodies for repeatable and reproducible results accepted in the scientific community. A human tubulogenesis assay developed by one of the co-authors of this Aop43, Dr. Tuula Heinonen (Finnish Centre for Alternative Methods, University of Tampere, Finland, now retired) is an OECD-validated test accepted in the regulatory community.

KE:28 Methods Response Summary: Box 6 shows the revised section on relevance and reliability of the methods with which KE:28 can be measured.

6. How it is Measure or Detected (KE:28, revised)

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 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 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].

R1.8 KE 110 – impairment of endothelial network

Comment: *DoA - various angiogenesis assays, including tubulogenesis in endothelial cells from zebrafish, chick, mouse and human species [Tal et al. 2017; Vargesson et al. 2003; Saili et al. 2019; McCollum et al. 2017; Nguyen et al. 2017, Zurlinden et al. 2020].*

Response: DoA text has been revised and updated to include these and other references relevant to endothelial network structure and function. This is particularly relevant for mapping the vast amounts of chemical effects data form ToxCast/Tox21 and other high-throughput screening efforts. The Aop43 has a hyperlink to a visual depiction of the process, where Aop43 can be placed in a broader context of cell-cell signaling systems that mediate endothelial tubule formation, maturation and stabilization using human cell-based data for *in vitro* profiling from the ToxCast high-throughput screening (HTS) dataset [Knudsen and Kleinstreuer, 2011], image accessed here:

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

7. KE:110 DoA (revised)

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].

Comment: *KE – description -Hanahan, 1997; Chung and Ferrara 2011; Coultas et al. 2005.*

Response: The impairment of endothelial networks ascribed to a disruption of blood vessel morphogenesis can be found in literature as 'patterned neovascularization'; however, that term is not recognized in the Gene Ontology lexicon. Instead, KE:110 best maps to the GO:0045446 'endothelial cell differentiation' (119 genes), defined as "*The process in which a mesodermal, bone marrow or neural crest cell acquires specialized features of an endothelial cell, a thin flattened cell. A layer of such cells lines the inside surfaces of body cavities, blood vessels, and lymph vessels, making up the endothelium*":

endothelial cell differentiation (119 genes)
blood vessel endothelial differentiation (9 genes)
cardiac endothelial cell differentiation (8 genes)
endothelial cell development (75 genes)
endothelial cell fate commitment (7 genes)
lymphatic endothelial cell differentiation (8 genes)
negative regulation of endothelial cell differentiation (11 genes)
positive regulation of endothelial cell differentiation (13 genes)
regulation of endothelial cell differentiation (44 genes)

As shown by the ontology, 97 (81.5%) of the 119 annotated genes map either to 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*". The subordinates for these categories are annotated as:

endothelial cell development (75 genes)

endocardial cell development (0 genes)
endothelial cell morphogenesis (17 genes)
establishment of blood-nerve barrier (5 genes)
establishment of endothelial barrier (52 genes)
negative regulation of endothelial development (2 genes)
positive regulation of endothelial development (8 genes)
regulation of endothelial cell development (19 genes)

regulation of endothelial cell differentiation (44 genes)
negative regulation of endothelial cell differentiation (11 genes)
positive regulation of endothelial cell differentiation (13 genes)

KE:110 description has been expanded with greater specificity and updated citations (see box 8, below), based on those concepts.

Comment: *How is it measured: Endothelial tubule formation Muller et al. 2002; Masckauchan et al. 2005; Sarkanen et al. 2010; Knudsen et al. 2016; Nguyen et al. 2016. Endothelial cells co-cultured with stromal cells: Bishop et al. 1999, and synthetic hydrogels: Nguyen et al. 2017*

Response: Some of that information may appear in earlier descriptions. Specific to KE:110, the measurement section has been expanded with more precise information and with updated citations (see box 9, below).

KE:110 Response Summary: Box 8 shows the revised KE:110 description and Box 9 the revised section on relevance and reliability of the methods with which KE:110 can be measured. Key points are as follows:

- Stressors: *potential vascular disrupting chemicals (pVDCs)*
- Taxonomic Applicability: *zebrafish, chick, mouse, rat, human*
- Lifestages: *embryonic development, organogenesis*
- Sex Applicability: *unspecific*

8. KE:110 Description (revised)

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].

9. How KE:110 is Measured or Detected (revised)

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 to 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.

R1.9 KE 298 – insufficiency, <Vascular>

Comment: *The consequences rather than the description of insufficiency are supported by (2) references: Therapontos et al. 2009; Vargesson et al. 2015.*

Response: The description to KE:298 has been re-written (see Box 10, below) to focus on the processes, rather than consequences (which are covered in AO:1001). The revised description includes an expanded discussion of modes-of-action of vascular insufficiency, including nutrient and oxygen supply to developing tissues and organs. Importantly, although the molecular toolbox is similar between zebrafish and mammalian embryos the small size of zebrafish embryos does not render them as vulnerable to hypoxia as vertebrate embryos, which become sensitive to hypoxia as embryos reach a size during organogenesis that cannot be effectively sustained by simple diffusion [Tron et al. 2007]. It includes an additional reference [Vargesson and Hootnick, 2017] that reviews how vascular insufficiency comes about experimentally and pathologically and reviews a variety of papers that detail this process in humans.

Comment: *How is it measured – by studying embryonic development and/or tissue cultures to determine effect of insufficiency (ie damage patterns). In embryo models – chick, rodent, zebrafish with GFP: Jin et al. 2005; Therapontos et al. 2009; Vargesson, 2015; Ellis-Hutchings et al. 2016; transgenic zebrafish embryos, live-cell imaging Clendenon et al. 2013; Shirinfard et al. 2013 and*

confocal cell imaging Tal et al. 2017; computational approaches… Kleinstreuer et al. 2011; Knudsen and Kleinstreuer, 2011

Response: The Methods for detecting KE:298 have been revised (see Box 11, below) to bring in more citations from the seminal work characterizing the effect of Thalidomide-induced limb defects eg, (phocomelia) on immature vascular networks of the early limb-bud. This work was performed in the lab of one of Aop43's co-authors (Dr. Vargesson). Several additional papers are cited [Beedie et al. 2015, 2016a and 2016b; Mahony et al. 2018; Beedie et al. 2020].

KE:298 Response Summary: Box 10 shows the revised KE:298 description and Box 11 the revised section on relevance and reliability of the methods with which KE:298 can be measured. Key points are as follows:

- Stressors: *developmental toxicants*
- Taxonomic Applicability: *zebrafish, chick, mouse, rat, human*
- Lifestages: *embryonic, development*
- Sex Applicability: *unspecific*

Box 10: KE:298 Description (*revised*)

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 of …. 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 were 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].

11. How KE:298 is Measured or Detected (revised)
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%) 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].

R1.10 AO 1001
Comment: *current knowledge that the specificity of the manifestations of embryo-fetal toxicity may vary greatly between species, and even between strains within the same species [Hurtt et al. 2003; Janer et al. 2008; Knudsen et al. 2009; Rorije et al. 2012; Theunissen et al. 2016].*

Response: no response requested from Reviewer #1; however, Reviewer #2 had some questions about AO:1001 (see comment R2.4 and our response and revised AO:1001).

R1.11 How is it measured

Comment: *OECD Test Guideline No. 414 (Prenatal Developmental Toxicity Study, OECD Test No. 415 (One-Generation Reproduction Toxicity Study) or Test No. 416 (Two-Generation Reproduction Toxicity)*

Response: No response needed. These guideline protocols have been referenced (see box 16 in the revised AO:1001 under comment R2.4, below.

R1.12 Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

Comment: *I don't know – cannot judge this without a more direct sense of the field*

Response: no response requested; more of the domain-specific comments were addressed in response to comments from Reviewers #2 and #3, which are addressed below.

R1.13 Is the weight-of-evidence judgement/scoring well described and justified based on the evidence presented? If not please explain.

Comment: *I think no – inconsistent relationships described; scoring not described or justified.*

Response: Revisions to AO:1001 in general and preceding KEs in particular address this reviewer comment. The increased precision and detail in the various KE descriptions show a clear flow of biological regulatory information as developmental angiogenesis progresses from disruption of VEGFR2 (MIE:305) to adverse developmental phenotypes (AO:1001). The rate incidence of developmental defects is an important consideration in any type of regulatory decision, whether the mechanisms are broad or specific. As such, AO:1001 will have links to many diverse MIEs. ***This reflects the common 'one-to-many' problem in the complex cascade of embryonic development: one MIE can lead to multiple AOs, and any AO maps to multiple MIEs.*** This concept is readily apparent in the thousands of curated gene-phenotype associations in the Mouse Genome Informatics database described earlier. We do believe consistency is conveyed by a focus on 'developmental angiogenesis', and that Aop43 has now been substantially improved thanks to the critical evaluation by our external reviewers.

R1.14 KER:335 Inhibition, VegfR2 leads to Reduction, Angiogenesis

Upstream event - Inhibition, VegfR2 (MIE:305); Downstream event - Reduction, Angiogenesis (KE:28).

Comment: *The specificity of the inhibitors includes activity on other receptors, eg. PDGFR. How can inhibition of EC migration and tumorigenic vessel formation have lower IC50 than receptor signal transduction inhibition? Possibly the sensitivity of the assay – also it's the same range and still rather similar values.*

Response: As the reviewer notes, crosstalk between VEGFR-2 and other pro-angiogenic receptor tyrosine kinase (RTK) activities such as PDGFR or FGFR is known. This 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). PDGFRβ and VEGFR-2 are closely related in the RTK kinome. Vatalanib inhibits both receptors but is most selective for VEGFR-2 [Wood et al. 2000].

In terms of synergistic inhibition of RTK inhibitors on tumorigenic vessel formation, it is not clear where the IC50 information cited by the reviewer came from. Epoxyquinol B inhibited kinase activity of several RTKs including VEGFR and PDGFR, and blocked VEGF-induced

migration and tubulogenesis in human umbilical vein endothelial cells (HUVECs) [Kamiyama et al. 2008]; Anlotinib inhibited cell migration and microvessel formation in the rat aortic ring assay and chicken chorioallantoic membrane assay via common downstream ERK signaling [Lin et al. 2018]; and Derazantinib at 0.1 µM to 3 µM blocked intersegmental vessel (ISV) migration linked to VEGF, PDGF, or FGF pathways in the zebrafish embryo [Kotini et al. 2020]. These examples are now cited in the revised KER:335 section (see box 13 below). The different assay platforms likely have different dynamic ranges that explain empirical differences.

Comment: *relationship between VEGFR2 signaling and angiogenic sprouting dynamics in human endothelial cells [Belair et al. 2016] and zebrafish embryos [Shirinifard et al. 2013].*

Response: Vatalanib suppressed zebrafish ISV migration at 0.07 µM [Tal et al. 2014] and at 0.01 µM inhibited angiogenic sprouting in a 3D human endothelial cell-based derived from induced pluripotent stem cells [Belair et al. 2016]. The Shirinifard et al. [2013] reference is a different context. In that study, high (likely environmentally unrealistic) concentrations of arsenic blocked ISV migration in the zebrafish embryo. Interestingly, exploratory filopodial behavior of EC-tip cells was increased by arsenic. Mathematical modelling inferred disruption of directional sensing of EC-tip cells leading to a more chaotic course of ISV outgrowth [Shirinifard et al. 2013]. The chaotic versus ordered dynamics is perhaps distal to RTK signal transduction, affecting cytoskeletal dynamics (actin, tubulin polymerization cycle) or interactions with the extracellular matrix (ECM). EC-tip cell migration is also sensitive to the urokinase-type plasminogen activator system (uPAR) during ECM remodeling [Beloglazova et al. 2021]. These details on angiogenic sprouting dynamics have been expanded in revised KER:335 (see box 12, below).

Comment: MOA not known *- 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****. But VEGFR2 is …master switch' for angiogenic sprouting [Herbert and Stanier 2011] only this reference for biological plaucibility of this KER?*

Response: The expanded description of KE:28 covers EC-tip versus EC-stalk selection in more detail, with additional references that support sprouting dynamics in this KER [Argraves et al. 2002; Williams et al. 2006; Eilken and Adams, 2010; Oladipupo et al. 2011; Blanco and Gerhardt, 2013; Venkatraman et al. 2016; Pauty et al. 2018; Beloglazova et al. 2021]. VEGF signals promote endothelial cell motility, filopodial extension and proliferation, and together with Notch signaling controls whether specific cells become leading EC-tip cells (non-proliferating) or trailing EC-stalk cells (proliferating). While VEGF-A promotes VEGFR-2 expression in EC-tip cells, the notch ligand, Dll4 suppresses VEGFR-2 expression in EC-stalk cells [Williams et al. 2006]. As such, complex VEGF-Notch/Dll4 signaling underlies the initial fate decision between EC-tip and EC-stalk selection as a part of the sphere of influence that VEGFR-2 activation has on the system.

Comment: *Empirical evidence supported by TG mice (VEGFA Ferrara et al. 1996; Carmellet et al. 1996, VEGFR1 Fong et al. 1995, VEGFR2 Shalaby et al. 1995), so VEGFR2 inhibition may lead to developmental defects also through disruption of other pathways unrelated to angiogenesis.... via cross talk with other receptors and pathways*

Response: We agree with this comment. Crosstalk between pro-angiogenic receptors on common downstream pathways like ERK signaling point to cell-specific expression of RTK density as critical determinants of target cell selectivity. For example, the endothelial TIE2 receptor is essential for ISV outgrowth in zebrafish [Li et al. 2014] and the angiopoietin/TIE2 receptor system at later stages [Zhang et al. 2021]. VEGF-dependent EC-tip cell migration is facilitated by the urokinase-type plasminogen activator receptor (uPAR), a signaling system

linked to cell-ECM interactions and Notch pathway components: Notch1 receptor and ligands (Dll1, Dll4, Jag1) in endothelial cells; and uPA, uPAR, TGFβ1, integrin β3, Jag1, Notch3 receptor in mural cells [Beloglazova et al. 2021]. That relationship is important for both endothelial network formation (KER:335) and network stabilization (KER:36) and has been added to the description of KER:335 (see box 12, below).

Overall Response for KER:335: The KER description has been more precisely discussed in the revised KER:335 Description (box 12) and Evidentiary support (box 13), transcribed below.

- Taxonomic Applicability: *zebrafish, rodent, human*
- Sex Applicability: *unspecific*
- Life Stage Applicability: *embryonic, development, pregnancy*

12. Key Event Relationship Description (*KER:335, revised*)

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 [EIlken 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].

13. Evidence Supporting this KER (*KER335, revised*)

Biological Plausibility: The control of EC-tip cell dynamics is a central feature linking VEGFR-2 inhibition (MIE:305) to adverse angiogenic sprouting behaviors (KE: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.

R1.15 KER 36 - Reduction, Angiogenesis leads to Impairment, Endothelial network

Upstream event – reduction, angiogenesis (KE:28), Downstream event – Impairment, Endothelial network (KE:110).

Comment: *I think this is backwards…. Because it starts from angiogenesis (the process) which is the result of endothelial network organisation. It would be more accurate if the terminology were specific to* ***angiogenic sprouting****, in which case it may be OK…*

Response: This has been more precisely explained above (see box 13) for *de novo* assembly of endothelial cells in the primitive capillary network of an early embryo (vasculogenesis) and an endothelial tubular network *in vitro* (tubulogenesis). Both are driven by

VEGF-A signaling, albeit with different target cells: angioblasts are the VEGF-responsive target cell in vasculogenesis but soon differentiate into endothelial cells that form the VEGF-Notch/Dll4 responsive target in angiogenesis.

In defining formal Gene Ontology semantics linked to 'endothelial cell development', genetic signals and responses can be found for at least 110 genes in the Mouse Gene Ontology Browser. These relationships connect KE:28 to upstream (MIE:305) and downstream (KE:110) events. They may be spatially distinct but are temporally fuzzy. Because the VEGF toolbox is similar for vasculogenesis and angiogenesis, the revised descriptions are retained but with greater transparency for the flow of biological information through Aop43 in detailing KER:36.

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

Response: This speaks to the previous comment and has been duly addressed for the regulation of spatio-temporal control of developmental angiogenesis. We provide an image (see below) for a ToxPi [Marvel et al. 2018] based profile of Aop43 developed for 21 assay targets 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]. The assay details were previously shown in a background section of Aop43 that somehow got labelled 'optional'. The details have been moved to KE:110 where they are now outlined. As a functional test, 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. This detail is better explained in KER:36.

Overall Response for KER:36: The KER description has been more precisely discussed in the revised KER:335 Description (box 14) and Evidentiary support (box 15), transcribed below.

- Taxonomic Applicability: *zebrafish, rodent, human*
- Sex Applicability: *unspecific*
- Life Stage Applicability: *all lifestages*

14. Key Event Relationship Description (*KER:36, revised*)

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].

15. Evidence Supporting this KER (*KER36, revised*)

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

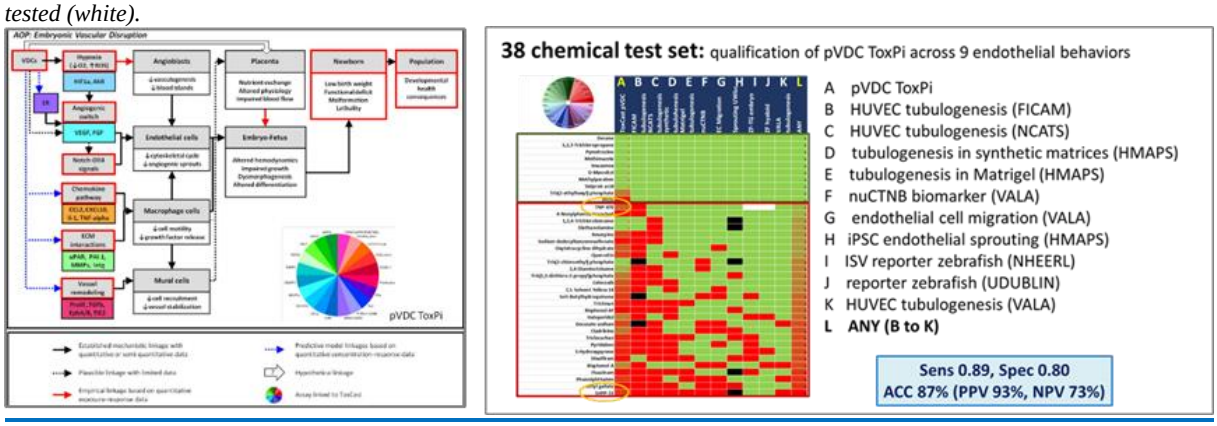
angiogenesis cycle (e.g., activation, sprouting, migration, tubulogenesis, vascular patterns). The ToxPi prediction was 87% accurate when in vitro observations were summed across all 10 platforms [Saili et al. 2019]. This shows the value of Aop43 in combining HTS data from ToxCast with biological knowledge 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).

Profiling Aop43 for predictive developmental vascular toxicity (pVDCs) in a battery of ToxCast assays that functionally map to genes curated for roles in developmental angiogenesis. LEFT: *ToxPi profile [Marvel et al. 2018] for 21 assay targets (slices) 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]. **RIGHT:** 38 ToxCast chemicals were independently tested by different laboratories using in vitro platforms sensitive to, or specific for, different stages in the angiogenesis cycle (e.g., activation, sprouting, migration, tubulogenesis, vascular patterns). The ToxPi prediction (column A) was 87% accurate when observations were summed across all 10 in vitro platforms (column L). Cells colors: non-active (green), active (red), cytotoxic (black), and not-tested (white).*



R1.16 KER:125 - Impairment, Endothelial network leads to Insufficiency, Vascular

Upstream event – Impairment, Endothelial network (KE:110), Downstream event – Insufficiency, Vascular (KE:298).

Comment: *weak support. The description refers to the next KER (developmental defects) <KER:1036>, not KER 126 <KER:125>. Husain et al. 2008, van Gelder et al. 2010, Gold et al. 2011, Thalidomide as empirical evidence.*

Response: Agreed. This comment was raised in more detail by Reviewer #3. KER:125 has been completely rewritten to address the biology linking KE:110 with KE:298. Reviewer #1 may refer to our response to comments from Reviewer #3 (R3.2 and box 17) for details.

R1.17 KER:1036 - Insufficiency, Vascular leads to Increased, Developmental Defects

Upstream event – insufficiency, vascular (KE:298), Downstream event – increased developmental defects (AO:1001).

Comment: KER 1036 *weak direct support. TG mice embryonic lethality is the empirical evidence of the AOP*

Response: Understood. A comment and its relationship to AO:1001 was raised in more detail by Reviewer #2. KER:1036 has been completely rewritten to address the biology linking KE:298 with AO:1001. Reviewer #1 should please refer to our response to comments from Reviewer #2 (R2.4 and box 16) for details.

Annex 2 – Section 2 - Reviewer #2

Thank you for these comments. A short response is given below to each comment. Please refer to detailed responses to Reviewer #1 comments, where we posted the proposed revised text for each element in 'boxes' that will be uploaded to Aop43, once the reviewers agree the comments and concerns have been satisfied.

R2.1 Comment: *The AOP43 has not been updated after 2015. Some references should be upgraded. I have traced them to some extent, but some cannot trace. Development of genetic research is rapid, so, the AOP43 should reflect current scientific information.*

Response: Please refer to our detailed response to comment R1.5. Our literature search with our PubMed AbstractSifter tool [Baker et al. 2017] was updated on November 30, 2021. The broad search returned 22,785 results that were reduced by automated and manual curation to 169 PubMed records, of which 76 were cited previously and 93 are new to this response.

R2.2. Comment: *As stressors, only two chemicals (vatalanib and Sunitinib malate Sunitinib (INN)) are described. If other one has found, please add.*

Response: Please refer to our detailed response to comment R1.5. Belair et al. (2016) evaluated 9 mechanistically diverse anti-angiogenic drugs in a human endothelial sprouting assay. The point-of-departure effect on anti-angiogenic potency followed the rank order: Vatalanib (10 nM) > Sunitinib malate (20 nM) > Combretastatin A4 (100 nM) > Temsirolimus (0.2 uM) > SB-3CT (0.5 uM) > Withaferin A (0.8 uM) > Thalidomide (2 uM), SU5416 (2 uM) > Nilotinib (7 uM). Therefore, Vatalanib represents the strongest stressor for MIE:305 followed in turn by Sunitinib and other mechanistically diverse compounds that are less specific or sensitive inhibitors of VEGFR2 activation.

R2.3 Comment: *AO, Data gaps; Taxonomic Applicability; Evidence in Rat is low. Toxicological studies in vivo are used rats, generally. If there are different pathway or different sensitivity between mice and rats for VEGFR signaling, we should know.*

Response: This is a good point, given the differential sensitivity of rodent versus nonrodent species to Thalidomide. I have not been able to find specifics on mouse versus rat embryos. This does not construe evidence that mouse and rat embryos are concordant in their sensitivity/specificity to VEGF-induced responses; however, three observations can be offered. (i) A commonly used goat polyclonal antibody to mouse VEGF detects rat VEGF in direct ELISAs and western blots but has low cross-reactivity with human VEGF (bio-techne cat# AF564, 22 citations); (ii) As pointed out in the revised Aop43 and in several publications by one of Aop43's co-authors (Dr. Neil Vargesson, University of Aberdeen) the concordance is strong for teratogenicity screening between zebrafish and chick (Beedie et al. 2016); and (iii) in Ellis-Hutchings et al. (2017), we observed concordant findings for two anti-angiogenesis chemicals (TNP-470, 5HPP-33) between rat WEC and zebrafish embryos, as well as in a human embryonic stem cell assay that is highly predictive of developmental toxicity in humans.

R2.4. Comment: *AO, Event1001 - Authors selected the four main types of developmental defects such as prenatal loss, malformations, low birth weight, and postnatal function. How did authors choose four events? Viability after delivery is also important event.*

Response: The four aspects are basic 'Principles of Teratology'. Quoting Friedman, 2010: The basic principles *"... have continued to guide scientific research in teratology, and they are*

widely used in teaching … and *… advances in our knowledge of the molecular and cellular bases of embryogenesis serve only to provide a deeper understanding of the fundamental developmental mechanisms that underlie Wilson's Principles of Teratology*".

The globally standardized protocol for prenatal developmental toxicity testing (OECD 414) is commonly an evaluation of rat and or rabbit fetuses just before term [Hurtt et al. 2003; Janer et al. 2008; Theunissen et al. 2016]. 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]. ***Defects involving major blood vessels (angiopathies) may be detected by some but represent a diagnostic challenge for assessing teratological effects in term fetuses.*** In contrast, some specific endpoints linked to anti-angiogenic teratogens such as phocomelia are easy to detect and measure can be [Therapontos et al. 2009; Beedie et al. 2016 and 2017]. Specific malformations do have considerable value for setting regulatory decisions of drugs and chemicals; however, they tend to show up less frequently in guideline studies.

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). These may be captured in a one-or two-generation reproduction toxicity study design (OECD 415 and 416, respectively). In many developing countries, the risk of these adverse outcomes is increased 10- to 100-fold higher than in the U.S. owing to inadequate healthcare systems and low levels of health expenditures [Goldenberg, 2004].

AO:1001 Response Summary: Box 16 shows the revised AO:1001, 'Increased, Developmental Defects'.

- Stressors: *developmental toxicants*
- Taxonomic Applicability: *mammals* (*rodents, non-rodents)*
- Lifestages: *embryonic, development, pregnancy*
- Sex Applicability: *unspecific*

16. AO:1001 Description (Increased, Developmental Defects, revised)

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: 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 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].

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).

R2.5 Comment: *Additional references to consider (excel file attached).*

Response: Thank you for these suggestions. Some are outdated from work in progress at the time and have since been published; all are cited and correctly referenced in the current revision of Aop43. On the spreadsheet provided below: 1, 16 (Ellis-Hutchings et al. 2017); 2, 3, 9, 12, 17 (Saili et al. 2019); 4, 13, 18 (McCollum et al. 2017); 5, 14 (Nguyen et al. 2017); 6, 11, 15, 19 (Tal et al. 2017); 7, 10 (Belair et al. 2016); 8 (Zurlinden et al. 2020); 20 (Theunissen et al. 2016) have all been cited. Some of that work was still in progress when Aop43 was under internal review and had not been updated for the summer 2021 external review (my apologies for that oversight).

No	頁	Title	Journal information	Memo
1	6	Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences.	Reprod Toxicol. 2017 Aug;71:16-31. doi: 10.1016/j.reprotox.2017.04.003. Epub 2017 Apr 13.	New title: Embryonic vascular disruption adverse outcomes: Linking high-throughput signaling signatures with functional consequences
2	6	RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences.	untraceable	in preparation
3	6	ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models.	untraceable	in preparation
4	6	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells	Reprod Toxicol. 2017 Jun;70:60-69. doi: 10.1016/j.reprotox.2016.11.005. Epub 2016 Nov 10.	
5	7	Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds.	untraceable	in preparation
6	7	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	Reprod Toxicol. 2017 Jun;70:70-81. doi: 10.1016/j.reprotox.2016.12.004. Epub 2016 Dec 19.	
7	9	Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays.	Acta Biomater. 2016 Jul 15;39:12-24. doi: 10.1016/j.actbio.2016.05.020. Epub 2016 May 13	
8	10	ToxCast HTS predictive model qualified by a validated human angiogenesis assay.	untraceable	in preparation
9	11	Identification of chemical vascular disruptors during development using an integrative predictive toxicity model and zebrafish and in vitro functional angiogenesis assays.	untraceable	in preparation
10	12	Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays.	Acta Biomater. 2016 Jul 15;39:12-24. doi: 10.1016/j.actbio.2016.05.020. Epub 2016 May 13	
11	12	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	See No.6	
12	14	ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models.	See No.3	
13	14	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells.	Reprod Toxicol. 2017 Jun;70:60-69. doi: 10.1016/j.reprotox.2016.11.005. Epub 2016 Nov 10.	
14	14	Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds.	See No.5	
15	14	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	See No.6	
16	16	Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences	See No.1	
17	16	RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences	See No.2	
18	16	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells.	See No. 13	
19	16	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity	See No.6	
20	18	Comparison of rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on the nature and severity of developmental effects.	Crit Rev Toxicol. 2016 Nov;46(10):900-910. doi: 10.1080/10408444.2016.1224807	

Annex 2 – Section 3 - Reviewer #3

Thank you for these comments. A short response is given below to each comment. Please refer to detailed responses to Reviewer #1 comments, where we posted the proposed revised text for each element in 'boxes' that will be uploaded to Aop43, once the reviewers agree the comments and concerns have been satisfied.

R3.1. Scientific quality

Comment: *Does the AOP incorporate all appropriate scientific literature and evidence? No, mayor models of developmental angiogenesis like the mouse retina model or the chicken embryo yolk assay (CAM) are missing. In many sections individual statements would need better referencing.*

Response: Please refer to our detailed response to comment R1.5. Our literature search with our PubMed AbstractSifter tool [Baker et al. 2017] was updated on November 30, 2021. The broad search returned 22,785 results that were reduced by automated and manual curation to 169 PubMed records, of which 76 were cited previously and 93 are new to this response.

In covering major models of developmental angiogenesis, we emphasized non-mammalian animal models due to their potential for high-throughput chemical testing strategies. My apologies for missing the neonatal mouse retina developmental model used to study endothelial cell guidance and subsequent formation of vascular patterns. Similarly, for the chick CAM assay now cited in KER:1036. KER:1036 connecting AE:298 (vascular impairment) to AO:1001 (developmental defects) was weak. It has been revised under guidance suggested by all three reviewers and appears below in response to comments and suggestions from Reviewer #3. Please refer to our response to R3.2 and box 17, below.

Comment: *Does the scientific content of the AOP reflect current scientific knowledge on this specific topic? Yes, but an update with more recent literature would be beneficial.*

Response: This has been completed as noted above.

R3.2. Weight of evidence (WoE)

Comment*: Is the weight-of-evidence judgement/scoring well described and justified based on the evidence presented? No. The WoE is mostly lacking any description and justification. A summarizing table as suggested in the Users Handbook (p51) on direct or indirect evidence is missing. All sections regarding Quantitative Understanding are missing.*

Response: The revised WoE sections have been extensively revised and updated (see the various boxes holding the proposed text for all revised sections of Aop43. We have not yet had time to complete the summarizing table suggested (p51) but will do so once all comments have been agreed to.

Comment: *Please consider weight-of-evidence for each Key Event Relationship (KER) and for the AOP as a whole. The AOP and its KERs are biologically plausible and well described in literature. I agree with the classifications as high and moderate.*

Response: The revised sections on evidentiary support for each KER and the AOP as a whole have been revised and updated (see the various boxes holding the proposed text for all revised sections of Aop43).

Comment: *The available data in the literature needs to be presented in a structured way, demonstrating quantitative relationships of MIE leading to AO. Especially the description of KE298 (Insufficiency, Vascular) needs improvement and it should be considered either changing the description to "Insufficiency, Blood Flow" or removing this KE altogether. Otherwise, its use should be justified in section "Essentiality of KEs" in regard of redundancy to KE110 (Impairment, Endothelial Network).*

Response: Gene Ontology nomenclature provides a structured way to better define the flow of biological regulatory information underlying quantitative relationships in Aop43. KE:298, which should be retained, has been substantially revised to provide justification and essentiality with regards to redundancy with KE:110 (see response to comment R1.9 and box 10).

In considering a potential name change of KE:298, as noted in our response to R1.9 and revised KE:298 (box 10) the revised description of KE:298 now focuses more properly on the processes leading to, rather than consequences resulting from, endothelial network impairment (which are covered in AO:1001). These processes include local regulation of blood flow, nutrient and oxygen availability that interact with morphoregulatory pathways. It includes an additional reference form one of the Aop43 co-authors [Vargesson and Hootnick, 2017] that reviews how vascular insufficiency comes about experimentally and pathologically and detail this process in humans.

On the other hand, the previous version of Aop43 failed to properly connect KE:110 (endothelial impairment) with KE:298 (vascular insufficiency). The fault lies in an improperly written KER:125. The information previously provided under KER:125 was out of place and belonged with the next KER:1036. This no doubt led to question whether KE:298 is needed in the first place. Revised KER:125 now more appropriately focuses on the functional diversification of endothelial networks leading to cardiovascular system morphology (box 17, transcribed below).

The Mammalian Phenotype Browser (MPO) defines 'abnormal blood vessel morphology' as "*any structural anomaly of the network of tubes that carries blood through the body*" [http://www.informatics.jax.org/vocab/mp_ontology/MP:0001614]. There are 3117 genotypes and 6341 annotations associated with this term. They consist of 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. This is obviously too comprehensive a term for any individual KER entity.

Drilling deeper into the MPO ontology, the term 'abnormal vascular development' defined as the "*aberrant process of vascular formation*" captures the biology relevant to KER:125 [http://www.informatics.jax.org/vocab/mp_ontology/MP:0000259]:

abnormal vascular development (1045 genotypes, 1768 annotations)
abnormal angiogenesis (721, 1119)
abnormal artery development (269, 441)
abnormal developmental vascular remodeling (96, 97)
abnormal physiological neovascularization (35, 35)
abnormal tumor vascularization (50, 50)
abnormal vascular branching morphogenesis (64, 65)
abnormal vein development (97, 124)

decreased angiogenesis (131, 131)
increased angiogenesis (47, 47)
abnormal blood vessel lumen formation (1, 1)
abnormal fetal ductus arteriosus morphology (45, 46)
absent fetal ductus arteriosus (6,6)
patent ductus arteriosus (34, 34)
premature closure of the ductus arteriosus (2,2)
abnormal perineural vascular complex morphology (5,5)
abnormal vascular endothelial cell development (46, 48)
abnormal vascular endothelial cell differentiation (7,7)
abnormal endothelial cell migration (25, 25)
abnormal vascular plexus formation (5, 5)
abnormal vascular smooth muscle development (1, 1)
abnormal vasculogenesis (82, 83)
absent organized vascular network (13,13)
abnormal vitelline vasculature morphology (357, 406)
abnormal vitelline artery morphology (3,3)
abnormal vitelline vein morphology (35, 42)
absent vitelline blood vessels (96, 96)
disorganized yolk sac venous plexus (30,30)

Once again complex, but this ontology level has relatively broad appeal for AOP elucidation and the subordinate terms are relevant to Aop43. It gives as sense of the controlled nomenclatures and evidence base that align neatly with the progression of Aop43 from KE:110 (endothelial networks) to KE:298 (vascular insufficiency). The next ontology level (not shown) identifies deeper parameters for different parts of the circulatory system and their remodeling as discussed in AE:298 (e.g., dorsal aorta, aoritc arches, intersomitic vessels, blood-brain barrier, blood supply to the retina, ...).

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

Upstream event – Impairment, Endothelial network (AE:110); Downstream event – Insufficiency, Vascular (AE:298)

- Taxonomic Applicability: *zebrafish, rodent, human*
- Sex Applicability: *unspecific*
- Life Stage Applicability: *embryo, development, pregnancy*

17. KER:125 (*revised*)

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: 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: 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: 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.

Quantitative Understanding of the Linkage

A number of anti-angiogenic compounds, including Vatalanib and Thalidomide, have been shown to quantitatively impair vascular patterning [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. 2016a; 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 [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 this KER in human studies is indirect, based solely on correlating malformations with vascular anatomy and/or developmental risks for women of reproductive potential or exposed during pregnancy to anti-angiogenic drugs [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]. These studies show the complexity of crosstalk between genetic signals and responses for vascular patterning versus morphoregulatory systems in general.

Domain of Applicability

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).

Response, KER:1036, Insufficiency, Vascular leads to Increased, Developmental Defects

Upstream event – Insufficiency, Vascular (AE:298); Downstream event – Increased, Developmental Defects (AO:1001)

The KER has been more precisely discussed in its Description and Evidentiary support (box 18), transcribed below.

- Taxonomic Applicability: *zebrafish, rodent, human*
- Sex Applicability: *unspecific*

- Life Stage Applicability: *embryo, development, pregnancy*

18. KER:1036 (*revised*)

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].

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: 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).

Quantitative Understanding of the Linkage

Concentration-dependent linkages reported for at least 9 anti-angiogenic compounds in chick limb and/or zebrafish embryos with regards to both vascular suppression and dysmorphogenesis [Tal et al. 2014; Beedie et al. 2016]. The general response on endothelial cells preceded effects on morphogenesis. Potential modulating factors include species susceptibility and stage dependency. Developmental buffering (canalization) systems may support resilience to exposure via angio-adaptative recovery mechanisms that are spatially and temporally differentiated.

Domain of Applicability

Wilson's Principles of Teratology (circa 1977) support the taxonomic applicability of teratogenesis. According to these long-standing Wilson's principles, the first on "Susceptibility to Teratogenesis Depends on the Genotype of the Conceptus and a Manner in which this Interacts with Adverse Environmental Factors". This principle has four main tenets: (i) species differences account for the fact that certain species respond to particular teratogens where others do not, or at least not to the same extent (e.g., humans and other primates are vulnerable to thalidomide induced phocomelia whereas rodents are not); (ii) strain and 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.

Comment: *Most KEs and KERs had essential sections missing completely or not used in alignment with the Guidance Document.*

Response: We have now completed all missing sections. Again, my apologies for the oversight. Some of that information was buried in other sections and got lost in translation.

Summary: *This AOP needs mayor revisions before it can be successfully applied.*

Response: We believe the revisions will bring Aop43 near ready for prime-time!

R3.3 AOP description[2]

p1, graphical representation: KERs should be included. These will be added during the editing, once responses/revisions have been approved.

p2, Background: The extensive information on angiogenic factors and pathways should be moved to the relevant sections of KEs and KERs **DONE**

[2] The page numbers indicated in this section refer to the AOP snapshot, available at: https://aopwiki.org/aopwiki/snapshot/pdf_file/43-2021-02-23T17:25:31+00:00.pdf

p3, KERs: There is no continuous path of adjacent KERs from MIE to AO as KER 125 and KER 1036 are designated non-adjacent. An oversight on our part. The previous description for KER:125 was actually to be in KER:1036; it is now corrected and expanded to include missing information.

p3, Stressors: Sunitinib malate Sunitinib (INN) should be changed to Sunitinib **DONE**

p4, Life stage applicability "conception < fetal" and "pregnancy" were chosen which only apply to mammals and not to selected taxa of zebrafish. Instead, "embryo" and "development" should be used as applicable life stage. **DONE.** Changes have been noted where appropriate; however, there are cases where the proper life stage is pregnancy, and that term has been added.

p4, Essentiality of the Key Events, an assessment of essentiality involving the 5 KEs of this AOP is missing **DONE**

Sections on Uncertainties and Inconsistencies, Quantitative Considerations, Assessment of Evidence Supporting the KERs, Review of Biological Plausibility of each KER, Review of Empirical Support for each KER and Review on Quantitative Understanding for each KER are missing. **DONE**

A summarizing table on tested stressors as suggested in the Users Handbook (p54) would be helpful. Due to the short turnaround time, we have gotten to this but will do so once the revisions have been approved.

R3.4 KE descriptions

In general: If these KEs are related to developmental stages only, this should be indicated in the Life Stage Applicability. If all life stages are discussed, examples for the role of VEGFR/angiogenesis in other areas like wound healing, inflammation, vascular remodeling or tumor growth should be included. How well models and methods apply to certain life stages should be discussed in section "Domain of Applicability". This has been clarified. Aop43 is specific to 'developmental angiogenesis' and the biology and toxicology applies mostly to organogenesis during stages covered by the OECD 414 guideline prenatal developmental toxicity study. As such, the 'lifestage' descriptor has been changed to "embryonic (organogenesis)" to distinguish it from utero-placental angiogenesis which would entail related but distinct AOPs.

MIE305, Inhibition, VegfR2

p8, How it is measured or detected **DONE.**

For more clarity in this section, the buildup of the AngioKB database should be removed as well as references to other pathways. It is mentioned that several assays directly measure capacity or bioactivity but only one example is given for each trait. Instead, all relevant assays measuring VEGFR2 capacity and bioactivity should be named directly. **DONE**

Zebrafish genetic homology should be discussed in section "Evidence for Biological Domain of Applicability". Application of zebrafish models expressing transgenes under VEGFR-promoters should be used with care in the context of this KE. As the authors state themselves, it is more useful to observe effects on angiogenesis and endothelial networks and less to directly measure inhibition of VEGFR2. Therefore, this method and other assessments of downstream consequences should be moved to the following KEs or their limitations as indirect assays carefully discussed. **DONE**

KE28, Reduction, Angiogenesis

P11, Level of Biological Organization, should be cellular instead of molecular **CORRECT – our mistake!**

P11, Domain of Applicability, the text here is not relevant for this section and should be moved to other sections **DONE.**

p11, Key Event description needs more description to define this event and distinguish it from KE305 and KE110. In the abstract of the AOP measurable events like altered cell fate and behavior of tip and stalk cells are mentioned. These should be included here. Ideally, the angiogenic state of a cell can be explained as a balance between pro- and anti-angiogenic signals. **DONE**

KE110, Impairment, Endothelial Network

p13, Key Event description needs careful revision, so not to impact on AOP150, but for better distinction from KE28. Differences of KE28 and KE110 should be discussed in the section for "Essentiality of KEs" of AOP43. **DONE**

KE298, Insufficiency, Vascular

p14, Level of Biological Organization, should be cellular or tissue instead of molecular **CORRECT**

p15, Domain of Applicability, the text here is not relevant for this section and should be moved to other "How it is measured", HTS should be spelled in full. **DONE**

p15, Key Event Description, needs better distinction from KE110. Differences of KE28 and KE110 should be discussed in the section for "Essentiality of KEs" of AOP43. Both KEs center on vascular disruption. I would suggest to focus instead in the description more on blood circulation and effective transport of oxygen, nutrients and removal of waste products as is suggested in the list of Key Event components (p14). That would also cover problems of unstable and leaky vessels in otherwise well-defined endothelial networks. Other problems of insufficient blood support, like slow or weak heartbeat, vessel occlusions or anemia should be mentioned. The point is on target; however, we believe it should be included under KER:125, which previously was incorrectly worded with what should have been in KER:1036 and, therefore, essentially missing from the Aop43. KER:125 is the link between endothelial network impairment (AE:110) and functional vascular insufficiency (AE:298). The new section sets the functional consequences of vascular impairment apart from structural development (morphogenesis).

p15, How it is measured, Methods unrelated to angiogenesis should be included, e.g. measuring oxygen saturation, blood velocity, blood pressure, blood flow patterns, erythrocyte cell counts, etc. This is not so straightforward in describing the flow of information leading to embryonic changes. There is literature on establishment and alterations in embryonic heart rate and yolk sac circulation from rodent embryo culture studies and clinical (sonogram) markers from human pregnancies that could be cited here, although they ae no definitive evidence. Perhaps the best example is establishment of the neurovascular unit (blood-brain-barrier, blood-retina-barrier).

AO1001, Increased, Developmental Defects

P16, Level of Biological Organization, should be organ or individual instead of molecular. **CORRECT**

P17 Domain of Applicability, the text here is not relevant for this section and should be moved to other sections. **DONE**

Section for the "Regulatory Significance of the AO" is missing. **DONE**

R3.5 KER descriptions

KER335, Inhibition VEGFR2 leads to Reduction, Angiogenesis

p18, For the Empirical Evidence a Table with Dose and Temporal Concordance would be useful. Due to the short turnaround time, we have gotten to this but will do so once the revisions have been approved.

P18, For Uncertainties and Inconsistencies it would be helpful to mention that both an increase on pro-angiogenic factors as well as a decrease in anti-angiogenic factors (notch signaling) can have similar outcomes. **DONE**

Missing sections: Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding) **DONE**

Quantitative Understanding is listed as "High" but the discussion on this classification is missing and no examples for quantitative readouts are given. **DONE**

KER36, Reduction, Angiogenesis leads to Impairment, Endothelial Network

p19, Key Event Relationship Description, in addition to angiogenesis the successful formation of an endothelial network requires stabilizing processes like anastomosis, lumen formation and remodeling like pruning. The role of non-endothelial cells like macrophages or pericytes involved in these processes should be mentioned. **DONE.** This appears at several places, but especially it is detailed under KE:298 (see comment R1.9, Box 10).

Missing sections: Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding). **DONE**

Quantitative Understanding is listed as "Moderate" but the discussion on this classification is missing and no examples for quantitative readouts are given. **DONE.**

KER125, Impairment, Endothelial Network lead to Insufficiency, Vascular

p20: Adjacency should be changed to adjacent. **DONE.**

It should be considered either to change this KER to "Impairment, Endothelial Network lead to Insufficiency, Blood Flow" or to remove this KE altogether. Instead a KER "Impairment, Endothelial Network lead to Increased, Developmental Defects" could be created. Reasons to keep this AOP organization should be discussed in "Essentiality of KEs". As noted above, there was a mixup in the previous description of KE:125 and KER:1036. This has been clarified in the revised versions of both KERs, as noted above in our response to Reviewer #3's comment.

KER1036, Insufficiency, Vascular lead to Increased, Developmental Defects

p21: Adjacency should be changed to adjacent Used different wording

Missing sections: KER Description, Biological Plausibility, Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding) Now provided

No	頁	Title	Journal information	Memo
1	6	Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences.	Reprod Toxicol. 2017 Aug;71:16-31. doi: 10.1016/j.reprotox.2017.04.003. Epub 2017 Apr 13.	New title: Embryonic vascular disruption adverse outcomes: Linking high-throughput signaling signatures with functional consequences
2	6	RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences.	untraceable	in preparation
3	6	ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models.	untraceable	in preparation
4	6	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells	Reprod Toxicol. 2017 Jun;70:60-69. doi: 10.1016/j.reprotox.2016.11.005. Epub 2016 Nov 10.	
5	7	Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds.	untraceable	in preparation
6	7	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	Reprod Toxicol. 2017 Jun;70:70-81. doi: 10.1016/j.reprotox.2016.12.004. Epub 2016 Dec 19.	
7	9	Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays.	Acta Biomater. 2016 Jul 15;39:12-24. doi: 10.1016/j.actbio.2016.05.020. Epub 2016 May 13	
8	10	ToxCast HTS predictive model qualified by a validated human angiogenesis assay.	untraceable	in preparation
9	11	Identification of chemical vascular disruptors during development using an integrative predictive toxicity model and zebrafish and in vitro functional angiogenesis assays.	untraceable	in preparation
10	12	Human iPSC-Derived Endothelial Cell Sprouting Assay in Synthetic Hydrogel Arrays.	Acta Biomater. 2016 Jul 15;39:12-24. doi: 10.1016/j.actbio.2016.05.020. Epub 2016 May 13	
11	12	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	See No.6	
12	14	ToxCast Model-Based Prediction of Human Vascular Disruption: Statistical Correlation to Endothelial Tubulogenesis Assays and Computer Simulation with Agent-Based Models.	See No.3	
13	14	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells.	Reprod Toxicol. 2017 Jun;70:60-69. doi: 10.1016/j.reprotox.2016.11.005. Epub 2016 Nov 10.	
14	14	Identification of a synthetic alternative to matrigel for the screening of anti-angiogenic compounds.	See No.5	
15	14	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity.	See No.6	
16	16	Embryonic vascular disruption: linking high throughput signaling signatures with functional consequences	See No.1	
17	16	RNA-Seq analysis of the functional-link between vascular disruption and adverse developmental consequences	See No.2	
18	16	Identification of vascular disruptor compounds by a tiered analysis in zebrafish embryos and mouse embryonic endothelial cells.	See No. 13	
19	16	Screening for chemical vascular disruptors in zebrafish to evaluate a predictive model for developmental vascular toxicity	See No.6	
20	18	Comparison of rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on the nature and severity of developmental effects.	Crit Rev Toxicol. 2016 Nov;46(10):900-910. doi: 10.1080/10408444.2016.1224807	

Annex 2 – Section 4 - Detailed Review of each chapter

AOP description

p1, graphical representation: KERs should be included **TBD**
p2, Background: The extensive information on angiogenic factors and pathways should be moved to the relevant sections of KEs and KERs **DONE**
p3, KERs: There is no continuous path of adjacent KERs from MIE to AO as KER 125 and KER 1036 are designated non-adjacent. **TBD**
p3, Stressors: Sunitinib malate Sunitinib (INN) should be changed to Sunitinib **DONE**
p4, Life stage applicability "conception < fetal" and "pregnancy" were chosen which only apply to mammals and not to selected taxa of zebrafish. Instead, "embryo" and "development" should be used as applicable life stage. **TBD**
p4, Essentiality of the Key Events, an assessment of essentiality involving the 5 KEs of this AOP is missing **DONE**
Sections on Uncertainties and Inconsistencies, Quantitative Considerations, Assessment of Evidence Supporting the KERs, Review of Biological Plausibility of each KER, Review of Empirical Support for each KER and Review on Quantitative Understanding for each KER are missing. **DONE**
A summarizing table on tested stressors as suggested in the Users Handbook (p54) would be helpful.**TBD**

KE descriptions

In general: If these KEs are related to developmental stages only, this should be indicated in the Life Stage Applicability. If all life stages are discussed, examples for the role of VEGFR/angiogenesis in other areas like wound healing, inflammation, vascular remodeling or tumor growth should be included. How well models and methods apply to certain life stages should be discussed in section "Domain of Applicability". **DONE.** Aop43 is specific to developmental angiogenesis and the biology and toxicology applies mostly to during early embryogenesis during stages covered by OECD414 guideline prenatal developmental toxicity study. As such, the 'lifestage' descriptor has been changed to "embryonic (organogenesis)" to distinguish it from utero-placental angiogenesis which would entail related but distinct AOPs.

MIE305, Inhibition, VegfR2

p8, How it is measured or detected **DONE.**
For more clarity in this section, the buildup of the AngioKB database should be removed as well as references to other pathways. It is mentioned that several assays directly measure capacity or bioactivity but only one example is given for each trait. Instead, all relevant assays measuring VEGFR2 capacity and bioactivity should be named directly. **DONE**
Zebrafish genetic homology should be discussed in section "Evidence for Biological Domain of Applicability". Application of zebrafish models expressing transgenes under VEGFR-promoters should be used with care in the context of this KE. As the authors state themselves, it is more useful to observe effects on angiogenesis and endothelial networks and less to directly measure inhibition of VEGFR2. Therefore, this method and other assessments of downstream consequences should be moved to the following KEs or their limitations as indirect assays carefully discussed. **DONE**

KE28, Reduction, Angiogenesis

P11, Level of Biological Organization, should be cellular instead of molecular **CORRECT – our mistake!**

P11, Domain of Applicability, the text here is not relevant for this section and should be moved to other sections **DONE.**
p11, Key Event description needs more description to define this event and distinguish it from KE305 and KE110. In the abstract of the AOP measurable events like altered cell fate and behavior of tip and stalk cells are mentioned. These should be included here. Ideally, the angiogenic state of a cell can be explained as a balance between pro- and anti-angiogenic signals. **DONE**

KE110, Impairment, Endothelial Network
p13, Key Event description needs careful revision, so not to impact on AOP150, but for better distinction from KE28. Differences of KE28 and KE110 should be discussed in the section for "Essentiality of KEs" of AOP43. **DONE**

KE298, Insufficiency, Vascular
p14, Level of Biological Organization, should be cellular or tissue instead of molecular **CORRECT**
p15, Domain of Applicability, the text here is not relevant for this section and should be moved to other "How it is measured", HTS should be spelled in full **DONE**

p15, Key Event Description, needs better distinction from KE110. Differences of KE28 and KE110 should be discussed in the section for "Essentiality of KEs" of AOP43. Both KEs center on vascular disruption. I would suggest to focus instead in the description more on blood circulation and effective transport of oxygen, nutrients and removal of waste products as is suggested in the list of Key Event components (p14). That would also cover problems of unstable and leaky vessels in otherwise well-defined endothelial networks. Other problems of insufficient blood support, like slow or weak heartbeat, vessel occlusions or anemia should be mentioned. **TBD**

p15, How it is measured, Methods unrelated to angiogenesis should be included, e.g. measuring oxygen saturation, blood velocity, blood pressure, blood flow patterns, erythrocyte cell counts, etc.**TBD**

AO1001, Increased, Developmental Defects
P16, Level of Biological Organization, should be organ or individual instead of molecular **CORRECT**
P17 Domain of Applicability, the text here is not relevant for this section and should be moved to other sections **TBD**
Section for the "Regulatory Significance of the AO" is missing. **TBD**

KER descriptions

KER335, Inhibition VEGFR2 leads to Reduction, Angiogenesis
p18, For the Empirical Evidence a Table with Dose and Temporal Concordance would be useful. **TBD**

P18, For Uncertainties and Inconsistencies it would be helpful to mention that both an increase on pro-angiogenic factors as well as a decrease in anti-angiogenic factors (notch signaling) can have similar outcomes. **TBD**

Missing sections: Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding) **TBD**
Quantitative Understanding is listed as "High" but the discussion on this classification is missing and no examples for quantitative readouts are given. **TBD**

KER36, Reduction, Angiogenesis leads to Impairment, Endothelial Network

p19, Key Event Relationship Description, in addition to angiogenesis the successful formation of an endothelial network requires stabilizing processes like anastomosis, lumen formation and remodeling like pruning. The role of non-endothelial cells like macrophages or pericytes involved in these processes should be mentioned. **TBD** – this appears at several places, but especially under KE:298 (see comment R1.9, Box 10)

Missing sections: Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding) **TBD**
Quantitative Understanding is listed as "Moderate" but the discussion on this classification is missing and no examples for quantitative readouts are given. **TBD**

KER125, Impairment, Endothelial Network lead to Insuffiency, Vascular
p20: Adjacency should be changed to adjacent **Used different wording**
It should be considered either to change this KER to "Impairment, Endothelial Network lead to Insufficiency, Blood Flow" or to remove this KE altogether. Instead a KER "Impairment, Endothelial Network lead to Increased, Developmental Defects" could be created. Reasons to keep this AOP organization should be discussed in "Essentiality of KEs".

KER1036, Insufficiency, Vascular lead to Increased, Developmental Defects
p21: Adjacency should be changed to adjacent **Used different wording**
Missing sections: KER Description, Biological Plausibility, Uncertainties and Inconsistencies, Quantitative Understanding (Response-response relationship, Time-scale, Known modulating factors, Known feedback loops influencing KER, Classification of quantitative understanding) **TBD**

Annex 3 : list of articles retrieved with Abstract Sifter tool and presented at the end of review TC

2019 Workflow for defining reference chemicals for assessing performance of in vitro assays. Judson RS, Thomas RS, Baker N, Simha A, Howey XM, Marable C, Kleinstreuer NC, Houck KA ALTEX 10.14573/altex.1809281

2018 ToxPi Graphical User Interface 2.0: Dynamic exploration, visualization, and sharing of integrated data models. Marvel SW, To K, Grimm FA, Wright FA, Rusyn I, Reif DM BMC bioinformatics 10.1186/s12859-018-2089-2

2018 The US Federal Tox21 Program: A strategic and operational plan for continued leadership. Thomas RS, Paules RS, Simeonov A, Fitzpatrick SC, Crofton KM, Casey WM, Mendrick DL ALTEX 10.14573/altex.1803011

2017 tcpl: the ToxCast pipeline for high-throughput screening data. Filer DL, Kothiya P, Setzer RW, Judson RS, Martin MT Bioinformatics (Oxford, England) 10.1093/bioinformatics/btw680

2017 Identification of vascular disruptor compounds by analysis in zebrafish embryos and mouse embryonic endothelial cells. McCollum CW, Conde-Vancells J, Hans C, Vazquez-Chantada M, Kleinstreuer N, Tal T, Knudsen T, Shah SS, Merchant FA, Finnell RH, Gustafsson JÅ, Cabrera R, Bondesson M Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2016.11.005

2017 Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium. Toimela T, Huttala O, Sabell E, Mannerström M, Sarkanen JR, Ylikomi T, Heinonen T Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2016.11.015

2017 Screening for angiogenic inhibitors in zebrafish to evaluate a predictive model for developmental vascular toxicity. Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A, McCollum CW, Bondesson M, Knudsen T, Padilla S, Kleinstreuer NReproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2016.12.004

2017 Embryonic vascular disruption adverse outcomes: Linking high throughput signaling signatures with functional consequences. Ellis-Hutchings RG, Settivari RS, McCoy AT, Kleinstreuer N, Franzosa J, Knudsen TB, Carney EW Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2017.05.005

2017 High-Content Assay Multiplexing for Vascular Toxicity Screening in Induced Pluripotent Stem Cell-Derived Endothelial Cells and Human Umbilical Vein Endothelial Cells. Iwata Y, Klaren WD, Lebakken CS, Grimm FA, Rusyn I Assay and drug development technologies 10.1089/adt.2017.786

2017 Versatile synthetic alternatives to Matrigel for vascular toxicity screening and stem cell expansion. Nguyen EH, Daly WT, Le NNT, Farnoodian M, Belair DG, Schwartz MP, Lebakken CS, Ananiev GE, Saghiri MA, Knudsen TB, Sheibani N, Murphy WL Nature biomedical engineering 10.1038/s41551-017-0096

2016 ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. Richard AM, Judson RS, Houck KA, Grulke CM, Volarath P, Thillainadarajah I, Yang C, Rathman J, Martin MT, Wambaugh JF, Knudsen TB, Kancherla J, Mansouri K, Patlewicz G, Williams AJ, Little SB, Crofton KM, Thomas RS Chemical research in toxicology 10.1021/acs.chemrestox.6b00135

2014 Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. Kleinstreuer NC, Yang J, Berg EL, Knudsen TB, Richard AM, Martin MT, Reif DM, Judson RS, Polokoff M, Dix DJ, Kavlock RJ, Houck KA Nature biotechnology 10.1038/nbt.2914

2013 Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays. Sipes NS, Martin MT, Kothiya P, Reif DM, Judson RS, Richard AM, Houck KA, Dix DJ, Kavlock RJ, Knudsen TB Chemical research in toxicology 10.1021/tx400021f

2013 High-content screening assay for identification of chemicals impacting cardiovascular function in zebrafish embryos. Yozzo KL, Isales GM, Raftery TD, Volz DC Environmental science & technology 10.1021/es403360y

2012 Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. Kavlock R, Chandler K, Houck K, Hunter S, Judson R, Kleinstreuer N, Knudsen T, Martin M, Padilla S, Reif D, Richard A, Rotroff D, Sipes N, Dix D Chemical research in toxicology 10.1021/tx3000939

2011 Activity profiles of 309 ToxCast™ chemicals evaluated across 292 biochemical targets. Knudsen TB, Houck KA, Sipes NS, Singh AV, Judson RS, Martin MT, Weissman A, Kleinstreuer NC, Mortensen HM, Reif DM, Rabinowitz JR, Setzer RW, Richard AM, Dix DJ, Kavlock RJ Toxicology 10.1016/j.tox.2010.12.010

2011 Environmental impact on vascular development predicted by high-throughput screening. Kleinstreuer NC, Judson RS, Reif DM, Sipes NS, Singh AV, Chandler KJ, Dewoskin R, Dix DJ, Kavlock RJ, Knudsen TB Environmental health perspectives 10.1289/ehp.1103412

2011 Predictive models of prenatal developmental toxicity from ToxCast high-throughput screening data. Sipes NS, Martin MT, Reif DM, Kleinstreuer NC, Judson RS, Singh AV, Chandler KJ, Dix DJ, Kavlock RJ, Knudsen TB Toxicological sciences : an official journal of the Society of Toxicology 10.1093/toxsci/kfr220

2010 Mining connections between chemicals, proteins, and diseases extracted from Medline annotations. Baker NC, Hemminger BM Journal of biomedical informatics 10.1016/j.jbi.2010.03.008

2010 In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, Reif DM, Rotroff DM, Shah I, Richard AM, Dix DJ Environmental health perspectives 10.1289/ehp.0901392

2010 Intra-Laboratory Pre-Validation of a Human Cell Based in vitro Angiogenesis Assay for Testing Angiogenesis Modulators. Sarkanen JR, Mannerström M, Vuorenpää H, Uotila J, Ylikomi T, Heinonen T Frontiers in pharmacology 10.3389/fphar.2010.00147

2009 Profiling bioactivity of the ToxCast chemical library using BioMAP primary human cell systems. Houck KA, Dix DJ, Judson RS, Kavlock RJ, Yang J, Berg EL Journal of biomolecular screening10.1177/1087057109345525

2004 Rapid structure-activity and selectivity analysis of kinase inhibitors by BioMAP analysis in complex human primary cell-based models. Kunkel EJ, Plavec I, Nguyen D, Melrose J, Rosler ES, Kao LT, Wang Y, Hytopoulos E, Bishop AC, Bateman R, Shokat KM, Butcher EC, Berg EL Assay and drug development technologies 10.1089/adt.2004.2.431

1999 An in vitro model of angiogenesis: basic features. Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF, Wheatley DN Angiogenesis 10.1023/a:1026546219962

2018 A Vascular Endothelial Growth Factor-Dependent Sprouting Angiogenesis Assay Based on an In Vitro Human Blood Vessel Model for the Study of Anti-Angiogenic Drugs. Pauty J, Usuba R, Cheng IG, Hespel L, Takahashi H, Kato K, Kobayashi M, Nakajima H, Lee E, Yger F, Soncin F, Matsunaga YT EBioMedicine 10.1016/j.ebiom.2017.12.014

2017 Vegfa signaling regulates diverse artery/vein formation in vertebrate vasculatures. Jin D, Zhu D, Fang Y, Chen Y, Yu G, Pan W, Liu D, Li F, Zhong TP Journal of genetics and genomics = Yi chuan xue bao 10.1016/j.jgg.2017.07.005

2016 Differential regulation of angiogenesis using degradable VEGF-binding microspheres. Belair DG, Miller MJ, Wang S, Darjatmoko SR, Binder BYK, Sheibani N, Murphy WL Biomaterials 10.1016/j.biomaterials.2016.03.021

2016 Human iPSC-derived endothelial cell sprouting assay in synthetic hydrogel arrays. Belair DG, Schwartz MP, Knudsen T, Murphy WL Acta biomaterialia 10.1016/j.actbio.2016.05.020

2014 Immediate and long-term consequences of vascular toxicity during zebrafish development. Tal TL, McCollum CW, Harris PS, Olin J, Kleinstreuer N, Wood CE, Hans C, Shah S, Merchant FA, Bondesson M, Knudsen TB, Padilla S, Hemmer MJ Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2014.05.014

2013 Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. Shibuya M Journal of biochemistry 10.1093/jb/mvs136

2012 Single-nucleotide polymorphisms in the KDR gene in pregnancies complicated by gestational hypertensive disorders and small-for-gestational-age infants. Andraweera PH, Dekker GA, Thompson SD, Roberts CT Reproductive sciences (Thousand Oaks, Calif.) 10.1177/1933719111428520

2008 Use of proximity ligation to screen for inhibitors of interactions between vascular endothelial growth factor A and its receptors. Gustafsdottir SM, Wennström S, Fredriksson S, Schallmeiner E, Hamilton AD, Sebti SM, Landegren U Clinical chemistry 10.1373/clinchem.2007.099424

2005 Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate. Yabu T, Tomimoto H, Taguchi Y, Yamaoka S, Igarashi Y, Okazaki T Blood 10.1182/blood-2004-09-3679

2005 BMP4 and noggin control embryonic blood vessel formation by antagonistic regulation of VEGFR-2 (Quek1) expression. Nimmagadda S, Geetha Loganathan P, Huang R, Scaal M, Schmidt C, Christ B Developmental biology 10.1016/j.ydbio.2005.01.005

2002 Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. Chan J, Bayliss PE, Wood JM, Roberts TM Cancer cell 10.1016/s1535-6108(02)00042-9

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. Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett 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, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G, Totzke F Cancer research

1999 Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW, Thomas KA The Journal of biological chemistry 10.1074/jbc.274.10.6453

1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW Nature 10.1038/380439a0

2018 CPS49-induced neurotoxicity does not cause limb patterning anomalies in developing chicken embryos. Mahony C, McMenemy S, Rafipay AJ, Beedie SL, Fraga LR, Gütschow M, Figg WD, Erskine L, Vargesson N Journal of anatomy 10.1111/joa.12712

2018 The syndrome of proximal femur, fibula, and midline metatarsal long bone deficiencies. Hootnick DR, Vargesson N Birth defects research 10.1002/bdr2.1349

2017 Arterial dysgenesis and limb defects: Clinical and experimental examples. Vargesson N, Hootnick DRReproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2016.10.005

2017 Regarding a limb with PFFD, fibular dimelia and mirror foot deformity.Hootnick DR, Vargesson N, Birch J Journal of pediatric orthopedics. Part B 10.1097/BPB.0000000000000490

2016 Congenital Embryonic Arterial and Skeletal Dysgeneses. Hootnick DR, DeSesso JM, Vargesson N Radiographics : a review publication of the Radiological Society of North America, Inc 10.1148/rg.2016150243

2016 Blood-brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. Fiorentino M, Sapone A, Senger S, Camhi SS, Kadzielski SM, Buie TM, Kelly DL, Cascella N, Fasano AMolecular autism 10.1186/s13229-016-0110-z

2015 Human vascular model with defined stimulation medium - a characterization study. Huttala O, Vuorenpää H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, Sarkanen JR, Heinonen T ALTEX 10.14573/altex.1411271

2014 Regulation of placental angiogenesis. Chen DB, Zheng J Microcirculation (New York, N.Y. : 1994) 10.1111/micc.12093

2014 Bidirectional crosstalk between periventricular endothelial cells and neural progenitor cells promotes the formation of a neurovascular unit. Vissapragada R, Contreras MA, da Silva CG, Kumar VA, Ochoa A, Vasudevan A, Selim MH, Ferran C, Thomas AJ Brain research 10.1016/j.brainres.2014.03.018

2013 Molecular parallels between neural and vascular development. Eichmann A, Thomas JL Cold Spring Harbor perspectives in medicine 10.1101/cshperspect.a006551

2011 Use of the mouse aortic ring assay to study angiogenesis. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B, Hodivala-Dilke K Nature protocols 10.1038/nprot.2011.435

2010 VEGF signaling has distinct spatiotemporal roles during heart valve development. Stankunas K, Ma GK, Kuhnert FJ, Kuo CJ, Chang CP Developmental biology 10.1016/j.ydbio.2010.08.030

2008 Descriptive epidemiologic features shared by birth defects thought to be related to vascular disruption in Texas, 1996-2002. Husain T, Langlois PH, Sever LE, Gambello MJ Birth defects research. Part A, Clinical and molecular teratology 10.1002/bdra.20449

2006 Prenatal exposure to thalidomide, altered vasculogenesis, and CNS malformations. Hallene KL, Oby E, Lee BJ, Santaguida S, Bassanini S, Cipolla M, Marchi N, Hossain M, Battaglia G, Janigro D Neuroscience 10.1016/j.neuroscience.2006.06.017

2004 The neural tube patterns vessels developmentally using the VEGF signaling pathway. Hogan KA, Ambler CA, Chapman DL, Bautch VL Development (Cambridge, England) 10.1242/dev.01039

2003 Vascularization of the developing chick limb bud: role of the TGFbeta signalling pathway. Vargesson N Journal of anatomy 10.1046/j.1469-7580.2003.00133.x

1998 Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M Proceedings of the National Academy of Sciences of the United States of America 10.1073/pnas.95.16.9349

2020 The complex TIE between macrophages and angiogenesis. Du Cheyne C, Tay H, De Spiegelaere W Anatomia, histologia, embryologia 10.1111/ahe.12518

2019 Standardized and Scalable Assay to Study Perfused 3D Angiogenic Sprouting of iPSC-derived Endothelial Cells In Vitro. van Duinen V, Stam W, Borgdorff V, Reijerkerk A, Orlova V, Vulto P, Hankemeier T, van Zonneveld AJ Journal of visualized experiments : JoVE 10.3791/59678

2017 Hif-1α regulates macrophage-endothelial interactions during blood vessel development in zebrafish. Gerri C, Marín-Juez R, Marass M, Marks A, Maischein HM, Stainier DYR Nature communications 10.1038/ncomms15492

2017 Integration of neurogenesis and angiogenesis models for constructing a neurovascular tissue. Uwamori H, Higuchi T, Arai K, Sudo R Scientific reports 10.1038/s41598-017-17411-0

2014 The zebrafish Tie2 signaling controls tip cell behaviors and acts synergistically with Vegf pathway in developmental angiogenesis. Li W, Chen J, Deng M, Jing Q Acta biochimica et biophysica Sinica 10.1093/abbs/gmu055

2013 A computational model predicting disruption of blood vessel development. Kleinstreuer N, Dix D, Rountree M, Baker N, Sipes N, Reif D, Spencer R, Knudsen T PLoS computational biology 10.1371/journal.pcbi.1002996

2008 Regulation of cell-cell adhesion by the cadherin-catenin complex. Nelson WJ Biochemical Society transactions 10.1042/BST0360149

2008 Three-dimensional analysis of vascular development in the mouse embryo. Walls JR, Coultas L, Rossant J, Henkelman RM PloS one 10.1371/journal.pone.0002853

2007 Endothelial cell migration during angiogenesis. Lamalice L, Le Boeuf F, Huot J Circulation research 10.1161/01.RES.0000259593.07661.1e

2005 Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. Masckauchán TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J Angiogenesis 10.1007/s10456-005-5612-9

2005 Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY Development (Cambridge, England) 10.1242/dev.02087

2002 Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Müller T, Bain G, Wang X, Papkoff J Experimental cell research 10.1006/excr.2002.5630

2002 Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocytic template and specific R-cadherin adhesion. Dorrell MI, Aguilar E, Friedlander M Investigative ophthalmology & visual science

2002 VEGF signaling is required for the assembly but not the maintenance of embryonic blood vessels. Argraves WS, Larue AC, Fleming PA, Drake CJ Developmental dynamics : an official publication of the American Association of Anatomists 10.1002/dvdy.10162

2000 Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. Patan S Journal of neuro-oncology 10.1023/a:1006493130855

1997 Signaling vascular morphogenesis and maintenance. Hanahan D Science (New York, N.Y.) 10.1126/science.277.5322.48

2015 Human vascular tissue models formed from human induced pluripotent stem cell derived endothelial cells. Belair DG, Whisler JA, Valdez J, Velazquez J, Molenda JA, Vickerman V, Lewis R, Daigh C, Hansen TD, Mann DA, Thomson JA, Griffith LG, Kamm RD, Schwartz MP, Murphy WL Stem cell reviews and reports 10.1007/s12015-014-9549-5

2015 Thalidomide (5HPP-33) suppresses microtubule dynamics and depolymerizes the microtubule network by binding at the vinblastine binding site on tubulin. Rashid A, Kuppa A, Kunwar A, Panda D Biochemistry 10.1021/bi501429j

2011 VEGF is essential for hypoxia-inducible factor-mediated neovascularization but dispensable for endothelial sprouting. Oladipupo S, Hu S, Kovalski J, Yao J, Santeford A, Sohn RE, Shohet R, Maslov K, Wang LV, Arbeit JM Proceedings of the National Academy of Sciences of the United States of America 10.1073/pnas.1101321108

2015 Vascular patterning regulates interdigital cell death by a ROS-mediated mechanism. Eshkar-Oren I, Krief S, Ferrara N, Elliott AM, Zelzer E Development (Cambridge, England) 10.1242/dev.120279

2014 Altered angiogenesis in low birth weight individuals: a role for anti-angiogenic circulating factors. Ligi I, Simoncini S, Tellier E, Grandvuillemin I, Marcelli M, Bikfalvi A, Buffat C, Dignat-George F, Anfosso F, Simeoni U 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 Obstetricians 10.3109/14767058.2013.807237

2011 A quantitative, facile, and high-throughput image-based cell migration method is a robust alternative to the scratch assay. Gough W, Hulkower KI, Lynch R, McGlynn P, Uhlik M, Yan L, Lee JA Journal of biomolecular screening 10.1177/1087057110393340

2011 Developmental and pathological angiogenesis. Chung AS, Ferrara N Annual review of cell and developmental biology 10.1146/annurev-cellbio-092910-154002

2010 Teratogenic mechanisms of medical drugs. van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N Human reproduction update 10.1093/humupd/dmp052

2009 Vascular endothelial growth factor receptor 2 (VEGFR-2) functions to promote uterine decidual angiogenesis during early pregnancy in the mouse. Douglas NC, Tang H, Gomez R, Pytowski B, Hicklin DJ, Sauer CM, Kitajewski J, Sauer MV, Zimmermann RC Endocrinology 10.1210/en.2008-1207

2009 Role of VEGF and tissue hypoxia in patterning of neural and vascular cells recruited to the embryonic heart. Liu H, Yang Q, Radhakrishnan K, Whitfield DE, Everhart CL, Parsons-Wingerter P,

Fisher SA Developmental dynamics : an official publication of the American Association of Anatomists 10.1002/dvdy.22103

2007 Tetralogy of fallot and alterations in vascular endothelial growth factor-A signaling and notch signaling in mouse embryos solely expressing the VEGF120 isoform. van den Akker NM, Molin DG, Peters PP, Maas S, Wisse LJ, van Brempt R, van Munsteren CJ, Bartelings MM, Poelmann RE, Carmeliet P, Gittenberger-de Groot AC Circulation research 10.1161/01.RES.0000261656.04773.39

1999 Role of vascular endothelial growth factor in the regulation of angiogenesis. Ferrara N Kidney international 10.1046/j.1523-1755.1999.00610.x

1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A Nature 10.1038/380435a0

1995 Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC Nature 10.1038/376062a0

2021 Molecular and Cellular Mechanisms of Vascular Development in Zebrafish. Eberlein J, Herdt L, Malchow J, Rittershaus A, Baumeister S, Helker CS Life (Basel, Switzerland) 10.3390/life11101088

2020 A cross-platform approach to characterize and screen potential neurovascular unit toxicants. Zurlinden TJ, Saili KS, Baker NC, Toimela T, Heinonen T, Knudsen TB Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2020.06.010

2020 Pericyte-Endothelial Interactions in the Retinal Microvasculature. Huang H International journal of molecular sciences 10.3390/ijms21197413

2019 Systems Modeling of Developmental Vascular Toxicity. Saili KS, Franzosa JA, Baker NC, Ellis-Hutchings RG, Settivari RS, Carney EW, Spencer R, Zurlinden TJ, Kleinstreuer NC, Li S, Xia M, Knudsen TB Current opinion in toxicology 10.1016/j.cotox.2019.04.004

2017 Vertebrate embryos as tools for anti-angiogenic drug screening and function. Beedie SL, Diamond AJ, Fraga LR, Figg WD, Vargesson N Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2016.11.013

2017 Blood-brain barrier development: Systems modeling and predictive toxicology. Saili KS, Zurlinden TJ, Schwab AJ, Silvin A, Baker NC, Hunter ES, Ginhoux F, Knudsen TB Birth defects research 10.1002/bdr2.1180

2016 Wnt Signaling in Cell Motility and Invasion: Drawing Parallels between Development and Cancer. Sedgwick AE, D'Souza-Schorey C Cancers10.3390/cancers8090080

2014 Techniques and assays for the study of angiogenesis. Irvin MW, Zijlstra A, Wikswo JP, Pozzi A Experimental biology and medicine (Maywood, N.J.) 10.1177/1535370214529386

2011 Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Herbert SP, Stainier DY Nature reviews. Molecular cell biology 10.1038/nrm3176

2011 Disruption of embryonic vascular development in predictive toxicology. Knudsen TB, Kleinstreuer NC Birth defects research. Part C, Embryo today : reviews 10.1002/bdrc.20223

2010 Dynamics of endothelial cell behavior in sprouting angiogenesis. Eilken HM, Adams RH Current opinion in cell biology 10.1016/j.ceb.2010.08.010

2009 Neurovascular development: The beginning of a beautiful friendship. Bautch VL, James JM Cell adhesion & migration 10.4161/cam.3.2.8397

2005 Angiogenesis in life, disease and medicine. Carmeliet P Nature 10.1038/nature04478

2005 Endothelial cells and VEGF in vascular development. Coultas L, Chawengsaksophak K, Rossant JNature 10.1038/nature04479

2012 Signaling required for blood vessel maintenance: molecular basis and pathological manifestations. Murakami M International journal of vascular medicine 10.1155/2012/293641

2007 The genetics of vasculogenesis. Drake CJ, Fleming PA, Argraves WS Novartis Foundation symposium 10.1002/9780470319413.ch6

2021 Anti-angiogenic activity of azathioprine. de Melo Bisneto AV, Fernandes AS, Velozo Sá VS, Véras JH, Soares ETS, da Silva Santos AF, Cardoso CG, Silveira-Lacerda EP, Carneiro CC, Chen-Chen L Microvascular research 10.1016/j.mvr.2021.104234

2020 Ex vivo model for studying endothelial tip cells: Revisiting the classical aortic-ring assay. Katakia YT, Duddu S, S N, Kumar P, Rahman F, Kumaramanickavel G, Chatterjee S Microvascular research 10.1016/j.mvr.2019.103939

2020 Robust and Scalable Angiogenesis Assay of Perfused 3D Human iPSC-Derived Endothelium for Anti-Angiogenic Drug Screening. van Duinen V, Stam W, Mulder E, Famili F, Reijerkerk A, Vulto P, Hankemeier T, van Zonneveld AJ International journal of molecular sciences 10.3390/ijms21134804

2020 Role of cereblon in angiogenesis and in mediating the antiangiogenic activity of immunomodulatory drugs. Beedie SL, Huang PA, Harris EM, Strope JD, Mahony C, Chau CH, Vargesson N, Figg WD FASEB journal : official publication of the Federation of American Societies for Experimental Biology 10.1096/fj.201903060RR

2020 Probing the Effects of the FGFR-Inhibitor Derazantinib on Vascular Development in Zebrafish Embryos.Kotini MP, Bachmann F, Spickermann J, McSheehy PM, Affolter M Pharmaceuticals (Basel, Switzerland) 10.3390/ph14010025

2019 Anti-Angiogenic Activity of Flunarizine by In Ovo, In Vitro, and In Vivo Assays. Kamili C, Kakaraparthy RS, Vattikuti UM Turkish journal of pharmaceutical sciences 10.4274/tjps.galenos.2018.29981

2018 Identification of Angiogenesis Inhibitors Using a Co-culture Cell Model in a High-Content and High-Throughput Screening Platform. Li S, Hsu CW, Sakamuru S, Zou C, Huang R, Xia M SLAS technology 10.1177/2472630317729792

2018 Anlotinib inhibits angiogenesis via suppressing the activation of VEGFR2, PDGFRβ and FGFR1. Lin B, Song X, Yang D, Bai D, Yao Y, Lu N Gene 10.1016/j.gene.2018.02.026

2018 Embryonic toxico-pathological effects of meglumine antimoniate using a chick embryo model. Khosravi A, Sharifi I, Tavakkoli H, Derakhshanfar A, Keyhani AR, Salari Z, Mosallanejad SS, Bamorovat M PloS one 10.1371/journal.pone.0196424

2016 Deleterious effects of tributyltin on porcine vascular stem cells physiology. Bernardini C, Zannoni A, Bertocchi M, Bianchi F, Salaroli R, Botelho G, Bacci ML, Ventrella V, Forni M Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 10.1016/j.cbpc.2016.03.001

2016 In vivo screening and discovery of novel candidate thalidomide analogs in the zebrafish embryo and chicken embryo model systems. Beedie SL, Rore HM, Barnett S, Chau CH, Luo W, Greig NH, Figg WD, Vargesson N Oncotarget 10.18632/oncotarget.8909

2016 Anti-Angiogenic Properties of Cafestol and Kahweol Palmitate Diterpene Esters. Moeenfard M, Cortez A, Machado V, Costa R, Luís C, Coelho P, Soares R, Alves A, Borges N, Santos A Journal of cellular biochemistry 10.1002/jcb.25573

2016 Shared mechanism of teratogenicity of anti-angiogenic drugs identified in the chicken embryo model. Beedie SL, Mahony C, Walker HM, Chau CH, Figg WD, Vargesson N Scientific reports 10.1038/srep30038

2015 Thalidomide-induced teratogenesis: history and mechanisms. Vargesson N Birth defects research. Part C, Embryo today : reviews 10.1002/bdrc.21096

2015 Effect of tributyltin on mammalian endothelial cell integrity. Botelho G, Bernardini C, Zannoni A, Ventrella V, Bacci ML, Forni M Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 10.1016/j.cbpc.2015.07.012

2015 Anticancer Properties of a Novel Class of Tetrafluorinated Thalidomide Analogues. Beedie SL, Peer CJ, Pisle S, Gardner ER, Mahony C, Barnett S, Ambrozak A, Gütschow M, Chau CH, Vargesson N, Figg WD Molecular cancer therapeutics 10.1158/1535-7163.MCT-15-0320

2013 3D quantitative analyses of angiogenic sprout growth dynamics. Shirinifard A, McCollum CW, Bolin MB, Gustafsson JÅ, Glazier JA, Clendenon SG Developmental dynamics : an official publication of the American Association of Anatomists 10.1002/dvdy.23946

2013 Pomalidomide is nonteratogenic in chicken and zebrafish embryos and nonneurotoxic in vitro. Mahony C, Erskine L, Niven J, Greig NH, Figg WD, Vargesson N Proceedings of the National Academy of Sciences of the United States of America 10.1073/pnas.1307684110

2012 Human adipose tissue extract induces angiogenesis and adipogenesis in vitro. Sarkanen JR, Kaila V, Mannerström B, Räty S, Kuokkanen H, Miettinen S, Ylikomi T Tissue engineering. Part A 10.1089/ten.TEA.2010.0712

2011 Methyl tert butyl ether targets developing vasculature in zebrafish (Danio rerio) embryos. Bonventre JA, White LA, Cooper KR Aquatic toxicology (Amsterdam, Netherlands) 10.1016/j.aquatox.2011.05.006

2010 Low-concentration tributyltin perturbs inhibitory synaptogenesis and induces neuronal death in immature but not mature neurons. Yamada J, Inoue K, Furukawa T, Fukuda A Toxicology letters 10.1016/j.toxlet.2010.07.011

2009 Decursin and decursinol angelate inhibit VEGF-induced angiogenesis via suppression of the VEGFR-2-signaling pathway. Jung MH, Lee SH, Ahn EM, Lee YM Carcinogenesis 10.1093/carcin/bgp039

2009 Malachite green induces cardiovascular defects in developing zebrafish (Danio rerio) embryos by blocking VEGFR-2 signaling. Jang GH, Park IS, Lee SH, Huh TL, Lee YM Biochemical and biophysical research communications 10.1016/j.bbrc.2009.01.118

2009 Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N Proceedings of the National Academy of Sciences of the United States of America 10.1073/pnas.0901505106

2009 Maternal administration of anti-angiogenic agents, TNP-470 and Angiostatin4.5, induces fetal microphthalmia. Rutland CS, Jiang K, Soff GA, Mitchell CA Molecular vision

2008 Epoxyquinol B shows antiangiogenic and antitumor effects by inhibiting VEGFR2, EGFR, FGFR, and PDGFR. Kamiyama H, Kakeya H, Usui T, Nishikawa K, Shoji M, Hayashi Y, Osada H Oncology research 10.3727/096504008784046063

2007 Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, Baranowski TC, Rubinstein AL, Doan TN, Dingledine R, Sandberg EM Cancer research 10.1158/0008-5472.CAN-07-3126

2005 Angiogenesis inhibitors derived from thalidomide. Noguchi T, Fujimoto H, Sano H, Miyajima A, Miyachi H, Hashimoto Y Bioorganic & medicinal chemistry letters 10.1016/j.bmcl.2005.08.086

1998 Oxygen, genes, and development: an analysis of the role of hypoxic gene regulation during murine vascular development. Maltepe E, Simon MC Journal of molecular medicine (Berlin, Germany) 10.1007/s001090050231

1995 Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats. Ema M, Kurosaka R, Amano H, Ogawa Y Journal of applied toxicology : JAT 10.1002/jat.2550150411

1990 Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J Nature 10.1038/348555a0

1968 A comparison of the teratogenic activity of thalidomide in rabbits and rats. Schumacher H, Blake DA, Gurian JM, Gillette JR The Journal of pharmacology and experimental therapeutics

1965 TERATOGENIC EFFECTS OF THALIDOMIDE IN RABBITS, RATS, HAMSTERS, AND MICE. FRATTA ID, SIGG EB, MAIORANA K Toxicology and applied pharmacology 10.1016/0041-008x(65)90095-5

2021 In vitro Three-Dimensional Sprouting Assay of Angiogenesis using Mouse Embryonic Stem Cells for Vascular Disease Modeling and Drug Testing. Galaris G, Thalgott JH, Teston E, Lebrin FPG Journal of visualized experiments : JoVE 10.3791/62554
2020 Defining an Upstream VEGF (Vascular Endothelial Growth Factor) Priming Signature for Downstream Factor-Induced Endothelial Cell-Pericyte Tube Network Coassembly. Bowers SLK, Kemp SS, Aguera KN, Koller GM, Forgy JC, Davis GE Arteriosclerosis, thrombosis, and vascular biology 10.1161/ATVBAHA.120.314517
2020 A simplified aortic ring assay: A useful ex vivo method to assess biochemical and functional parameters of angiogenesis. Kapoor A, Chen CG, Iozzo RV Matrix biology plus 10.1016/j.mbplus.2020.100025
2019 Perfused 3D angiogenic sprouting in a high-throughput in vitro platform. van Duinen V, Zhu D, Ramakers C, van Zonneveld AJ, Vulto P, Hankemeier T Angiogenesis 10.1007/s10456-018-9647-0
2016 Flt-1 (VEGFR-1) coordinates discrete stages of blood vessel formation. Chappell JC, Cluceru JG, Nesmith JE, Mouillesseaux KP, Bradley VB, Hartland CM, Hashambhoy-Ramsay YL, Walpole J, Peirce SM, Mac Gabhann F, Bautch VL Cardiovascular research 10.1093/cvr/cvw091
2009 Distinct role of PLCbeta3 in VEGF-mediated directional migration and vascular sprouting. Bhattacharya R, Kwon J, Li X, Wang E, Patra S, Bida JP, Bajzer Z, Claesson-Welsh L, Mukhopadhyay D Journal of cell science 10.1242/jcs.041913
2009 Local guidance of emerging vessel sprouts requires soluble Flt-1. Chappell JC, Taylor SM, Ferrara N, Bautch VL Developmental cell 10.1016/j.devcel.2009.07.011
2008 FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Fischer C, Mazzone M, Jonckx B, Carmeliet P Nature reviews. Cancer 10.1038/nrc2524
2004 The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL The American journal of pathology 10.1016/S0002-9440(10)63711-X
1995 Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Fong GH, Rossant J, Gertsenstein M, Breitman ML Nature 10.1038/376066a0
2021 DPSCs treated by TGF-β1 regulate angiogenic sprouting of three-dimensionally co-cultured HUVECs and DPSCs through VEGF-Ang-Tie2 signaling. Zhang Y, Liu J, Zou T, Qi Y, Yi B, Dissanayaka WL, Zhang C Stem cell research & therapy 10.1186/s13287-021-02349-y
2021 Mesenchymal stromal cells enhance self-assembly of a HUVEC tubular network through uPA-uPAR/VEGFR2/integrin/NOTCH crosstalk. Beloglazova I, Stepanova V, Zubkova E, Dergilev K, Koptelova N, Tyurin-Kuzmin PA, Dyikanov D, Plekhanova O, Cines DB, Mazar AP, Parfyonova Y Biochimica et biophysica acta. Molecular cell research 10.1016/j.bbamcr.2021.119157
2018 New models to study vascular mural cell embryonic origin: implications in vascular diseases. Sinha S, Santoro MM Cardiovascular research 10.1093/cvr/cvy005
2018 Evaluating the potential of endothelial cells derived from human induced pluripotent stem cells to form microvascular networks in 3D cultures. Bezenah JR, Kong YP, Putnam AJ Scientific reports 10.1038/s41598-018-20966-1
2018 Incorporating Pericytes into an Endothelial Cell Bead Sprouting Assay. Azam SH, Smith M, Somasundaram V, Pecot CV Journal of visualized experiments : JoVE 10.3791/57309
2016 Computational Screening of Tip and Stalk Cell Behavior Proposes a Role for Apelin Signaling in Sprout Progression. Palm MM, Dallinga MG, van Dijk E, Klaassen I, Schlingemann RO, Merks RM PloS one 10.1371/journal.pone.0159478
2016 Time to Decide? Dynamical Analysis Predicts Partial Tip/Stalk Patterning States Arise during Angiogenesis. Venkatraman L, Regan ER, Bentley K PloS one 10.1371/journal.pone.0166489
2009 Identification of small molecule compounds that inhibit the HIF-1 signaling pathway. Xia M, Bi K, Huang R, Cho MH, Sakamuru S, Miller SC, Li H, Sun Y, Printen J, Austin CP, Inglese J Molecular cancer 10.1186/1476-4598-8-117

2008 New tubulin polymerization inhibitor derived from thalidomide: implications for anti-myeloma therapy. Kizaki M, Hashimoto Y Current medicinal chemistry 10.2174/092986708783955473
2006 Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. Williams CK, Li JL, Murga M, Harris AL, Tosato G Blood 10.1182/blood-2005-03-1000
2005 Tubulin-polymerization inhibitors derived from thalidomide. Inatsuki S, Noguchi T, Miyachi H, Oda S, Iguchi T, Kizaki M, Hashimoto Y, Kobayashi H Bioorganic & medicinal chemistry letters 10.1016/j.bmcl.2004.10.072
2000 The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. Yeh JR, Mohan R, Crews CM Proceedings of the National Academy of Sciences of the United States of America 10.1073/pnas.97.23.12782
1997 Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC Nature 10.1038/386403a0
2000 Placental defects in ARNT-knockout conceptus correlate with localized decreases in VEGF-R2, Ang-1, and Tie-2. Abbott BD, Buckalew AR Developmental dynamics : an official publication of the American Association of Anatomists 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1080>3.0.CO;2-N
2021 Physiopathology of late-onset fetal growth restriction. Araujo Júnior E, Zamarian AC, Caetano AC, Peixoto AB, Nardozza LM Minerva obstetrics and gynecology 10.23736/S2724-606X.21.04771-7
2021 Genetic Basis and Therapies for Vascular Anomalies. Queisser A, Seront E, Boon LM, Vikkula M Circulation research 10.1161/CIRCRESAHA.121.318145
2016 Comparison of rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on the nature and severity of developmental effects. Theunissen PT, Beken S, Beyer BK, Breslin WJ, Cappon GD, Chen CL, Chmielewski G, De Schaepdrijver L, Enright B, Foreman JE, Harrouk W, Hew KW, Hoberman AM, Hui JY, Knudsen TB, Laffan SB, Makris SL, Martin M, McNerney ME, Siezen CL, Stanislaus DJ, Stewart J, Thompson KE, Tornesi B, Van der Laan JW, Weinbauer GF, Wood S, Piersma AH Critical reviews in toxicology 10.1080/10408444.2016.1224807
2012 Relative parameter sensitivity in prenatal toxicity studies with substances classified as developmental toxicants. Rorije E, van Hienen FJ, Dang ZC, Hakkert BH, Vermeire T, Piersma AH Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2012.05.092
2011 Anatomic and etiological classification of congenital limb deficiencies. Gold NB, Westgate MN, Holmes LB American journal of medical genetics. Part A 10.1002/ajmg.a.33999
2010 The principles of teratology: are they still true? Friedman JM Birth defects research. Part A, Clinical and molecular teratology 10.1002/bdra.20697
2009 Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U.S. EPA's ToxRefDB. Knudsen TB, Martin MT, Kavlock RJ, Judson RS, Dix DJ, Singh AV Reproductive toxicology (Elmsford, N.Y.) 10.1016/j.reprotox.2009.03.016
2008 A retrospective analysis of developmental toxicity studies in rat and rabbit: what is the added value of the rabbit as an additional test species? Janer G, Slob W, Hakkert BC, Vermeire T, Piersma AH Regulatory toxicology and pharmacology : RTP 10.1016/j.yrtph.2007.11.007
2004 Factors influencing perinatal outcomes. Goldenberg RL Annals of the New York Academy of Sciences 10.1196/annals.1315.032
2003 Proposal for a tiered approach to developmental toxicity testing for veterinary pharmaceutical products for food-producing animals. Hurtt ME, Cappon GD, Browning A Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 10.1016/s0278-6915(02)00326-5
2002 Recurrent miscarriage: aetiology, management and prognosis. Li TC, Makris M, Tomsu M, Tuckerman E, Laird S Human reproduction update 10.1093/humupd/8.5.463