# Vertebrate embryos as tools for anti-angiogenic drug screening and function

Shaunna Beedie[1,2], Alexandra J. Diamond[1], Lucas Rosa Fraga[1], William D. Figg[2], Neil Vargesson[1,*]

1 School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK.

2 Molecular Pharmacology Section, Genitourinary Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health. Bethesda. USA.

*Corresponding author
*Neil Vargesson:* *n.vargesson@abdn.ac.uk; nvargesson@gmail.com*

Key words: angiogenesis, chicken, zebrafish, mouse, rat, rabbit, non-human primates, thalidomide

Abstract

The development of new angiogenic inhibitors highlights a need for robust screening assays that adequately capture the complexity of vessel formation, and allow for the quantitative evaluation of the teratogenicity of new anti-angiogenic agents. This review discusses the use of screening assays in vertebrate embryos, specifically focusing upon chicken and zebrafish embryos, for the detection of anti-angiogenic agents.

## Introduction and background

The cardiovascular system is vital for normal embryonic development *in utero* [1]. It is one of the earliest differentiating and functioning organ systems, emphasizing its importance to the embryo [2-7]. The primitive vascular system develops by vasculogenesis, *de novo* differentiation and growth of vessels from the mesoderm. The major vessels in the embryo form first, the dorsal aortae (transporting oxygenated blood from the placenta or yolk sac to the heart) and vena cava or vitelline veins (transporting deoxygenated blood back to the heart or yolk sac) develop by vasculogenesis. Expansion of the nascent vascular network can then occur by angiogenesis, the process of vessel formation from the preexisting vasculature. This process also vascularizes newly forming tissues and organs including the developing limbs [8]. Angiogenesis itself is composed of several physiological processes; endothelial sprouting, intussusceptive angiogenesis and arteriogenesis (the recruitment of smooth muscle to the vessel). These processes are discussed in more detail in the following section. The angiogenic process is not just limited to embryogenesis. Indeed, angiogenesis is vital for menstruation, and wound healing and repair, in the adult. Additionally, inappropriate angiogenesis is a hallmark of some pathological conditions [9]. Tumour progression, for example, is generally dependent on the formation of a vascular supply (although recent studies have shown vessel co-option is an alternative survival method during tumour development) [10, 11]. The newly formed vasculature supplies nutrients, removes waste products, and enables tumour growth, and the promotion of metastasis [9]. The vasculature is

therefore a novel alternative target for the development of new therapeutic agents. *In vitro* angiogenic assays are limited in their ability to predict new compound action, while mouse models are not appropriate for high throughput screening. Within this review, we discuss the angiogenic process, and how *in vivo* systems can more accurately depict angiogenesis than *in vitro* assays. The benefits, and drawbacks, of using animal systems for investigating normal physiological angiogenesis, and the screening and development of new agents is explored. We specifically focus on the benefits of small animal systems, and their increasing use in drug screening laboratories. In addition, we use the anti-angiogenic, teratogenic pharmaceutical agent thalidomide, as an example of a complex agent that can be successfully evaluated in these model systems.

## Angiogenesis in health and disease

The precursor to developmental angiogenesis is embryonic vasculogenesis. This process begins when the angioblasts from within blood islands (clusters of angioblasts), are induced to aggregate by vascular endothelial growth factor A (VEFGA) and neuropilin-1 (expressed on the arterial epithelium). The culmination of this event is the formation of the primitive vascular plexus (Figure 1). VEGF is an inducer of angiogenesis, and an essential factor required for endothelial cell survival. Intricate VEGF signaling from the endoderm and mesoderm, then promotes the conversion of the primitive vascular plexus to a vascular network [12]. The vascular network is extensively remodeled, and following this the vessels are stabilized by mural cells, including pericytes (Figure 1). This process is regulated by platelet-derived growth factor B (PDGFB) and transforming growth factor β (TGF- β) signaling. The onset of blood vessel formation is controlled by hypoxia inducible factor (HIF). HIF-1 binds to hypoxia response elements within the promoters and enhancers of target genes associated with angiogenic growth factors and glucose metabolism. These include VEGF (Figure 2), PDGF-β, transforming growth factor beta (TGF- β), angiopoietin (Ang2), inducible nitric oxide synthetase (iNOS), insulin-like growth factor 2 (IGF-2), adrenomedullin, epidermal growth factor (EGF), and urokinase-type plasminogen

activator [13]. The combination of increased expression of angiogenic and regulatory transcription factors leads to increased blood flow and thus increased oxygen delivery [9]. The VEGF, and VEGF receptor system, are best characterized, and provide a target for many anti-angiogenic therapies [14] (Figure 3). As well as being directed by the expression of growth factors, vessels themselves express regulatory signaling molecules to non-vascular cells during development. The importance of the vasculature in development is therefore two fold, in addition to providing oxygen for tissue development, blood vessels express cues fundamental for cell fate specification, embryonic patterning, organ differentiation and tissue remodeling. The importance of angiogenesis during embryonic development has been shown in multiple mouse knock out models, where a loss of genes involved in angiogenesis, such as c-Myc [15], are terminally fatal [16, 17]. Loss of VEGF [18] or VE-cadherin [19] results in abnormal vascular development, while loss of HIF-1α [20] or neuropilin [21] causes perturbed vascular remodeling.

Moreover, vascular malformations, mispatterning or perturbed vascular remodeling in the embryo have been associated with skeletal and other morphological defects in a number of human disorders. For example, increased prevalence of vascular anomalies are seen in individuals with Schimmelpenning syndrome, characterised by craniofacial and skeletal abnormalities [22]. Defects caused by vascular disruption, the interruption or prevention of development of fetal vasculature, can be a result of disrupted or destroyed embryonic structures that would otherwise have developed normally. The type of fetal structural defects resulting from vascular disruption depend on the areas affected, usually those with most sensitive peripheral vasculature [23]. Limb defects, such as clubfoot and limb reduction syndromes, are quite often associated with vascular disruption or a failure to produce the correct vascular patterns [24]. Cleft palate and craniofacial malformations are also reported.

During adulthood, angiogenesis remains important for wound healing, tissue and organ functions, and the menstrual cycle. Angiogenesis is also essential for tumor growth. Recent years have seen an increase in the use of anti-angiogenic agents to treat cancerous and non-cancerous conditions, where inappropriate angiogenesis causes disease progression; including ophthalmic diseases [25, 26], Crohns disease [27, 28], as anti-obesity agents [9, 29] and have also shown promise in endometriosis [30-32]. An increased incidence in prescribing drugs to treat these conditions requires a thorough understanding of the effects of these drugs on the vasculature (Figure 3), as well as possibly the introduction of pregnancy prevention programs, for example, the Risk Evaluation and Mitigation Strategy (REMS) program used with the prescription of the teratogenic agents thalidomide, lenalidomide, and pomalidomide [33]. The REMS program is a regulated distribution program, which aims to reduce the risk of embryo-fetal exposure to teratogenic compounds, and informs prescribers, pharmacists and patients on the serious risks and safe-use conditions for these compounds.

Understanding the mechanisms and/or effects of drugs upon angiogenesis is therefore essential to determine effectiveness in treatment of pathological conditions like cancer, but also to ensure their use is avoided during pregnancy.

Animal models to study anti-angiogenic drugs and their actions

To predict the effects or potential of new drugs upon human embryonic health, drugs are tested in pregnant animal (usually mammalian) models. Due to their rapid development, embryos are highly sensitive to teratogen exposure. Laboratory animals can be treated according to specific dosing schedules, and their offspring can be assessed for anatomical and physiological abnormalities. However, a perplexing aspect of the pharmaceutical agent thalidomide, complicates the development of screening assays. Thalidomide, and some other drugs (such as valproic acid), exhibits species specificity in its actions [34, 35]; for example, thalidomide does not cause congenital abnormalities in certain strains of mice [36]. When originally prescribed in the 1950s, thalidomide was

presumed to be safe for use during pregnancy. However, it became clear that thalidomide was detrimental to embryonic development, and over 10,000 children were born with defects due to thalidomide exposure. Because of the thalidomide tragedy, new therapeutic agents must be screened in at least two different animal models, typically a rodent and a non-rodent model, before being approved for use [36-38].

Testing in large numbers of animals can be both time consuming and expensive. However, small animals, such as zebrafish and chicken embryos, limit the number of large mammalian animals required for initial screening, and allow for the testing of a large number of compounds [39-42]. Such assays allow a preliminary understanding of the toxicity and pharmacology and could elucidate molecular mechanisms underlying the drug action. Mammals, such as non-human primates (NHPs) and rodents, are more representative of the effect on human development. However, in an age where the use of pregnant animals in research is becoming increasingly regulated, and the public perception of animal use, puts pressure on reducing the number of mammals used in research, the use of embryos from lower species to provide preliminary information is becoming increasingly useful. Consequently, the development of new, cost-effective systems for the efficient and sensitive detection of hazardous agents is vital. The following sections describe the uses, benefits, and hindrances, of using zebrafish, chicken, mouse, rabbit and NHPs for the use of screening for anti-angiogenic agents, and understanding the angiogenic process.

-Zebrafish (*Danio rerio*)

Unlike mammals, which can be costly to keep, zebrafish are relatively inexpensive. Embryos develop externally from the mothers, and develop rapidly, and the optical transparency of the tissues permits *in vivo* observation of angiogenesis and organ morphology. Vasculogenesis and angiogenesis in the zebrafish have been well characterized for the purpose of studying vascular physiology [40, 43-46] and for drug screening [41, 47-54]. Like other vertebrates,

the development of the zebrafish cardiovascular system begins with the differentiation of precursor cardiovascular cells from the lateral mesoderm. At approximately 12 hours post-fertilization (hpf), the cells of the mesoderm express SCL/Tal-1 and Flk1, beginning the differentiation of the cells to hemangioblasts. The original vessel in the vertebrate trunk (the dorsal aorta) forms by vasculogenesis. Subsequent intersegmental vessel (ISV) formation occurs by sprouting from the dorsal aorta at approximately 24 hpf. The sprouts migrate between the somites to anastomose and culminate in the formation of the dorsal longitudinal anastomic vessel. Blood vessels in the zebrafish are similar to those of other vertebrates; the vasculature is a single cell layer of endothelial cells, with smooth muscle cells, pericytes and fibroblasts supporting the structure [55]. Like other vertebrates, molecular regulation of vessel morphogenesis involves the VEGF ligands and their receptors [56], BMPs [57], and intrinsic cell-cell communication [58].

The zebrafish has been successfully utilized as a model system for screening compounds that alter angiogenesis for over 15 years [40, 51, 59]. The zebrafish can be exposed to the compounds early in development, then fixed and stained at a set time point. Changes in the vasculature can be quantified by assessing the number of forming intersomitic vessels, and by measuring the extent of their outgrowth. Anti-angiogenic agents may reduce the number, or length of outgrowth of these vessels. Additionally, because angiogenesis is essential for organ development, the effects of compounds can be compared based on the observational outcome. Past studies have quantified eye growth, pectoral fin outgrowth and otic vesicle development as markers for anti-angiogenic action, as well as effects on spinal development (for example, curved or twisted spines) and a general restriction in the growth of the body size [5, 60]. While these measurements can be easily made in the laboratory by imaging each treated zebrafish, several studies have optimized this process for high throughput screening. Robotic systems can automatically position zebrafish, and composite images can be created by imaging layers of the translucent zebrafish embryo

[39]. The zebrafish embryo assay has major advantages; hundreds of compounds can be quickly screened in multiwell plates, zebrafish development is fast and allows for rapid output of results and the highly characteristic and hardwired vessel patterning means the assessment of effects is straightforward compared to higher animals [54, 60].

One of the key advantages of the zebrafish embryo is the ability to image embryos *in vivo* and throughout development. Another key advantage of the zebrafish is the ability to generate tissue-specific transgenic lines, in particular one that allows visualization of the vasculature throughout development and effects of compounds upon the process [61]. The use of the transgenic *fli1*:EGFP zebrafish allows for the imaging of blood vessels and to observe the effects of drugs in living tissue (Figure 4A, B). *fli1* is a well established endothelial cell marker, meaning transgene expression of the enhanced green fluorescent protein (EGFP) is tissue specific [61]. Development of the naïve vessels can be tracked and quantified, and the extent of outgrowth can be used as a reliable *in vivo* assessment of angiogenesis [50-52, 54, 62-67]. This can also be translated to high throughput screening, for example, by reading fluorescent intensity in the living zebrafish [68].

The translational ability of the efficacy and toxicity of anti-angiogenic drugs exposed to *fli1*:EGFP zebrafish has been established. By screening a range of well characterized angiogenesis inhibitors, Chimote *et al* [62] demonstrated that the zebrafish assay could differentiate anti-angiogenic compounds acting by VEGFR inhibition (such as sunitinib), and those acting by non-selective cytotoxicity, like TNP-470, a Met-AP2 inhibitor (Figure 3). Similar results have been seen regarding vascular development in anti-angiogenic compound treated zebrafish embryos [54]. The anti-angiogenic monoclonal antibodies (such as bevacizumab) did not reduce vessel outgrowth (likely due to lack of humanized antibody binding to the zebrafish ortholog) demonstrating the assays ability to separate drugs by mechanism. The results correlated well with other *in vitro* and

*in vivo* assays [54, 62]. Confirmation of the activity of compounds in the zebrafish assay, has streamlined the preclinical drug discovery process [50-52]. Due to their size, the zebrafish is easily converted to high-throughput screening assays [39]. For researchers interested in cancer development and cancer angiogenesis, the adult zebrafish is amenable to cancer cell grafts. Similar *in vivo* imaging assays can be used to assess the effect of anti-angiogenic therapies on the tumor vasculature [69].

Zebrafish embryos are also useful for deciphering molecular mechanisms of action, and to elucidate causes of teratogenesis. Thalidomide has been shown to mediate anti-angiogenic teratogenic actions by depleting VEGF receptors [70] leading to reduced filopodial extension and tube formation [64]. Thalidomide has been shown to reduce pectoral fin outgrowth in zebrafish [52, 71]. Zebrafish with non-functional cereblon do not produce the fin defects when exposed to thalidomide, suggesting that thalidomide-induced teratogenicity may occur by binding to the cereblon complex [71]. Since this finding, several clinical reports have shown a correlation between cereblon expression and the response to thalidomide, and its analogs lenalidomide and pomalidomide in the adult condition, multiple myeloma [72-75].

-Chicken

The chicken embryo is a well-established model in experimental embryology due to its accessibility to micromanipulation techniques during organogenesis stages [76-79]. The chicken embryo is increasingly also being used for drug screening and understanding how anti-angiogenic drugs act [50-52, 54, 64, 80-83].

By using standard toxicology testing end points, including gross anatomy development, reductions in size/mass of cartilage elements and eye size, the chicken embryo can be used to rapidly assess the teratogenicity of new compounds. For example, the chicken embryo has been used to assess the teratogenic actions of the anti-epileptic drug, Valproic acid [84, 85]. Valproic acid

has been used in the treatment of epilepsy, psychiatric disorders, migraines, and in preventing seizures [86]. Recent research has also implicated its efficacy in the treatment of AIDS and cancer (phase 2 clinical trials), due to the drug's ability to inhibit histone deacetylase [86]. Exposure of the human embryo to valproic acid can lead to harm to the developing embryo and is commonly referred to as fetal valproate syndrome, where damage includes spina bifida, and cranio-facial defects [87, 88]. In the developing chicken embryo, valproic acid has been shown to be anti-angiogenic and teratogenic [84], producing a pattern of defects similar to those in humans including neural and cardiac defects [89] and limb reduction defects [84]. Several mechanisms have been proposed to explain the teratogenic effect of valproic acid including reduced embryonic folate metabolism [90], hypoxia induced vascular insults [91] and alterations in retinoid metabolism [92, 93].

The chicken embryo has also been utilized to study thalidomide-induced defects since the 1960s [94, 95]. The limb defects produced in chickens exposed to thalidomide during development do share some similarities to those seen in human thalidomide embryopathy cases [64, 94-98]. Indeed, thalidomide exposure can result in damage to the axial artery supplying the forming forelimbs with blood [96]. More recently studies have shown thalidomide can induce cell death [97] and changes to the vessel networks in the chorioallantoic membrane (CAM), that supplies nutrients from the yolk to the embryo [99] and CPS49, an anti-angiogenic analog of thalidomide, can cause a time-sensitive range of limb reduction anomalies [64, 98]. Further studies have shown that the chicken embryo can also be used to identify anti-angiogenic, teratogenic thalidomide analogs [50-52] and teratogenic risk of anti-angiogenic compounds [54]. The molecular mechanisms the drug influences to cause defects has also been explored using the chicken embryo, showing that many important signals involved in limb outgrowth and patterning, including Fgf8, Fgf10, Shh, BMP and Wnt signaling, is altered in limb buds exposed to thalidomide [97, 100] and to CPS49 [64].

In addition to utilizing the embryo itself, the surrounding yolk sac membrane (YSM) and the chorioallantoic membrane (CAM) can be used to demonstrate a compounds anti-angiogenic activity or potential [54, 82, 83, 101, 102]. The YSM and the CAM are extraembryonic membranes formed during chicken embryo development. The YSM develops early around HH St10 and is the first site where blood vessels form from angioblasts (i.e. vasculogenesis) which then spread all over the yolk (through angiogenesis) and supplies nutrients to the embryo. The CAM forms from day 4 and is a fusion of the allantois and the chorionic membrane. The CAM is attached to the embryo, and exponentially increases in size as the embryo develops. Its function is to remove waste products. In these bioassays, a sponge, bead, or gel soaked in the compound of interest is placed upon the YSM or CAM, and left for a designated period of time. At the end point, the vessels can be quantified using imaging or colourmetric analysis [83]. The YSM and CAM assay is therefore useful for rapid investigation of compounds, but the number of eggs required to perform the assay may limit its use. In summary, the chicken embryo model can be used to evaluate angiogenesis, teratogenic risk, and the molecular effects of drugs (Figure 4C-D).

-Rodents

The use of mice as experimental models is well documented. Around 95% of our DNA is shared with that of a mouse [103], and most of the shared diseases are caused by the same genetic changes [104]. During mouse embryonic development, angioblast cells generated in blood islands at E7.0 integrate in the embryonic mesoderm post-gastrulation to develop the vascular plexus at around E8.5 [105-107]. The connection of the embryonic vasculature with the yolk sac vasculature is thought to take place between E8.25-E9.0 [108]. Through angiogenesis, extensive remodelling and recruitment of the pericytes and smooth muscle to the vascular wall, the primitive network is developed and matured [105]. Crucial signalling factors during blood vessel development include

VEGF,the previously discussed signaling factor essential for angiogenesis [109-111]. Recruitment of pericytes and smooth muscle to form mature blood vessels is regulated by the angiopoietin/Tie2 system. In response to angiopoietin/Tie2 interaction, endothelial cells release platelet-derived growth factor (PDGF) to attract local mesenchymal cells, which then differentiate in to pericytes and smooth muscle cells [112, 113].

Angiogenesis in mice is often studied in relation to cancer, since angiogenesis is required for tumour growth [114]. Folkman, Haudenschild and Zetter [115] isolated the first angiogenic and anti-angiogenic factors from animal tumours [115]. These early studies lay the foundations for development of anti-angiogenic drugs for the treatment of cancer. The most common procedure used to analyse angiogenesis in tumour growth in adult mice is the transplantation of a tumour cell line in to immune deficient mice [116]. Anti-angiogenic drugs, for example soluble receptors or neutralising antibodies, have been developed for cancer treatment utilising mouse tumour models.

Adult mouse models have also been utilised to study conditions such as ischemia, where restricted blood supply to a particular area of the body can cause tissue damage. A mouse model of hindlimb ischemia was made by ligation and excision of the femoral artery, which demonstrated that neovascularisation, characterised by increased capillary density and endothelial cell proliferation, was dependent on up-regulation of VEGF expression. VEGF expression was increased up to 14 days after ischemia was induced compared to non-operated limbs. Indeed, neutralising VEGF protein prevented neovascularisation in the ischemic limb, therefore demonstrating VEGF is a key regulator of angiogenesis [117]. Mice and rat models of myocardial infarction are also used to evaluate drug treatments, since the neovascularisation involved in wound healing after a myocardial infarction is very efficient [118].

An advantage of the mouse is the ability to make transgenic tissue reporter lines. Though embryo development can not be followed live and *in vivo* like the zebrafish and chicken, the ability to make tissue specific reporter lines also allows tissue specific knockdown to test gene function. For example, angiogenesis has been visualized during embryonic development through utilising mouse strains expressing β-galactosidase gene in endothelial cells [119-122] Two receptor tyrosine kinase (RTK) families are nearly exclusively expressed by endothelial cells: vascular endothelial growth factor receptors (VEGFR1-3) and Tie receptors (Tie1-2). VEGFR-1 acts primarily to restrict angiogenesis, and VEGFR-2 and VEGFR-3 act to stimulate angiogenesis of vascular and lymphatic endothelial cells, respectively [123]. The platelet-derived growth factor (PDGF) and Eph receptor tyrosine kinases are also important during angiogenesis, and are required for stabilization of vascular walls and in characterization of arterial versus venous vessels, respectively [124, 125]. Fong et al. [119] used targeted mutations at the Fms-related tyrosine kinase 1 (Flt-1) locus (*flt-1*$^{lcz}$), encoding the VEGFR-1 protein, to determine that Flt-1 is necessary for organisation of vasculature during embryogenesis [119]. Tie1-2 receptors and angiopoietin ligands exhibit context dependent roles in endothelial cell survival and in remodeling of the vessel networks [126]. Puri et al. [120] generated *Tie-1*$^{lcz}$(-/-) mice to show that receptor tyrosine kinase TIE-1 is essential to maintain integrity of vascular endothelial cells [120].

Mice are fundamental for angiogenesis research, but unfortunately mice can respond differently upon exposure to anti-angiogenic compounds compared to humans [127]. Perhaps the best known example is thalidomide where mice and rats appear to be insensitive or less sensitive to thalidomide with several studies reporting no skeletal abnormalities [128-130] though in some strains of mice thalidomide-induced damage has been observed [131, 132]. Interestingly thalidomide exposure in late pregnancy in rats has been shown to cause brain damage in areas associated with autism in humans, and has been linked to blood vessel loss [133]. Explanations for the apparent resistance of rodents to

thalidomide include that rodents may produce more efficient antioxidants against free radicals, thalidomide has a shorter half-life in rodents and does not appear to induce apoptosis as it does in humans [134-136]. Another factor may be differences in metabolism and clearance rates of the drug [36, 137-139]. Indeed, a recent paper using a transgenic mouse line where CYP3 metabolic enzymes were missing from the placenta resulted in mouse embryos with some thalidomide-induced defects [140]. In terms of anti-cancer drugs to treat tumours or tumour grafts in the animal, rodents do show strain-differences in their ability to respond to these compounds. This may be a result of implantation sites of tumour cells (frequently xenografts are subcutaneous) [127, 141] and the speed of cancer growth since tumour developmental processes can take weeks in mice and years in humans [142]. Crucially, there is also wide genetic variation between humans who develop cancers, which is not reflected when using experimental mouse models bred from strains of identical backgrounds [127]. While incredibly useful, developing rodent embryos are not as readily manipulated or as accessible as zebrafish or chicken embryos, so consequently *in vitro* assays are used to test the effect of anti-angiogenic factors. The maintenance is more costly, and *in vivo* imaging becomes much more invasive than with smaller animal models.

-Rabbits

Rabbits are one of the most commonly used non-rodent species in toxicological testing, after mice and rats [143]. They are relatively easy to maintain and breed and have short gestation periods. However, development of the embryo and effects of drugs *in vivo* are difficult to observe as embryonic development occurs in the uterus. Developmental toxicity studies have been performed on rabbit embryos to screen agents such as drugs (eg: thalidomide) and industrial compounds [144, 145]. In addition whole mount embryo cultures can be useful in order to analyse the effect of compounds upon embryo development [144, 146]. A retrospective study comparing rat and rabbit *in vivo* studies, showed similar

sensitivity between rats and rabbits in drug screens, and perhaps explains why rats are more widely used to test drug action [145]. In contrast, Theunissen [147] demonstrated some species differences between rat and rabbit in the development of embryo-fetal developmental toxicity, for example fetal malformations were more commonly seen in the rat, whereas embryo-fetal death and embryo resorption was more prominent in the rabbit [147]. Taken altogether, these studies show that selective toxicity and species differences in testing of developmental toxicity is fairly common.The use of both rodent and non-rodent species is suggested as more beneficial in detecting developmental toxicants compared to the use of one species alone [147, 148].

Many of the studies in vasculogenesis and angiogenesis performed in rabbit are carried out in adult animals. Rabbit corneal pocket assay is used to test the antiangiogenic capacity of determined compounds. One important example of the use of this technique was the landmark paper by D'Amato and colleagues [149]. Within this study, the authors promoted angiogenic outgrowth in the rabbit eye using FGF pellets, and subsequently observed which agents were able to reduce or inhibit the angiogenic outgrowth. Using this method, the authors became the first group to demonstrate thalidomide`s anti-angiogenic properties. They hypothesized upon the use of thalidomide as an anti-angiogenic therapeutic, and proposed that the anti-angiogenic actions may be responsible for the teratogenic effects [149].

-Non-human primates

Non-human primates (NHPs) are more similar to humans than the other mammalian models, and so likely provide a more relevant understanding of angiogenesis and drug action [150]. Indeed, studies have been performed to investigate the role of vascularisation and angiogenesis during early pregnancy and development, and the molecules that control these processes. For example,

in baboons, oestrogen controls the expression of VEGF in placental vascularization [151].

NHPs particularly the common marmoset (*Callithrix jacchus*) and cynomolgus monkey (*Macaca fascicularis*) have been used to assess the teratogenic potential of drugs including those with anti-angiogenic and anti-cancer properties [152, 153]. One of the more widely studied drugs in NHPs is thalidomide. Cynomolgus monkey's following thalidomide exposure exhibit forelimb and hindlimb defects, as well as craniofacial defects, tail and genital organ defects [154]. In addition, the defects caused by thalidomide can vary widely among the offspring from severe, where all limbs are effected to mild where just the tail appears abnormal [154-156]. Similarly, a study analysing the potential teratogenic effects of a potent thalidomide analog EM-12 (2-(2,6-dioxopiperidine-3-yl)-phthalimidine) in the common marmoset resulted in embryos presenting a range of defects similar to humans, among them forelimb and hindlimb defects (amelia, phocomelia), defects on mandibular, palate, ribs and tail [155, 156]. Further analysis suggested cell adhesion molecule changes occur following EM-12 exposure and have been proposed to be a potential target of thalidomide embryopathy [157, 158]. Furthermore, in Cynomolgus monkey fetuses following exposure to thalidomide, thousands of gene changes were identified, many of the genes related to actin cytoskeletal remodelling and genes involved in vasculature development [154]. A down-regulation of vascular pathways may effect vascular cell proliferation and recruitment in developing limbs and lend support to previous proposals suggesting limb defects are secondary to disruption in blood vessel development [37, 98, 136, 154].

Thalidomide is also a potent anti-inflammatory agent [35, 37]. A high level of crosstalk is observed between the vascular and inflammatory pathways. Yet although down-regulation of inflammatory genes may reflect a protective response to thalidomide, down-regulation of genes in both pathways by the drug

suggests the effects of thalidomide are likely seen across a range of regulatory processes [37, 154, 159].

When and how drugs cause effects in the forming NHP embryo is unclear. As the embryo develops *in utero*, studies to follow the effects in real time are very difficult particularly in comparison to zebrafish and chicken embryos, where general effects on angiogenesis and embryonic development can be seen and followed with relative ease. Once the effects of drugs/compounds in smaller animals are known, later studies in NHPs can shed light on other effects or be used to check agents safety in a more human relevant model.

Summary

The formation of a functional blood supply by vasculogenesis and angiogenesis is a required precursor to the development of the cardiovascular system during embryogenesis. Few data are available on the safety of anti-angiogenic drugs when given during pregnancy; although factors such as mechanism, dose, bioavailability, and developmental stage must be considered, accumulating evidence suggests that anti-angiogenic drugs as a class may be teratogenic [54]. Anti-angiogenic compounds are contraindicated during pregnancy due to the disruption of the delicate embryonic vasculature. Most anti-angiogenic compounds carry a FDA category C (for example bevacizumab) or D (for example sunitinib) warning, indicating that adverse events are seen when drugs are taken during fetal development but studies are limited. A demand still exists for thorough and reliable methods for the detection of teratogens, particularly identification of anti-angiogenic drugs. A well-integrated understanding of the intricate development of the vasculature and new methods of drug screening may offer opportunities for therapeutic intervention and identifications of mechanisms of teratogenicity. Small animal models are incredibly useful in the early stages of preclinical drug development. However, reproductive toxicology studies are usually conducted late in the drug development process, and unexpected results are costly. Animal studies do not obviate concerns related to teratogens that are uniquely harmful to humans and thus extrapolation of data

obtained from non-mammalian models must be done with caution. Nonetheless, the ability to accurately predict a drugs action reduces the number of large animals required, and increases the safety profile of new drugs. Although we discuss *in vivo* developmental models, which encapsulate more of the angiogenic process than an *in vitro* culture system, knowledge gained from *in vitro* experiments are incredibly useful, and should not be negated. At present, combining *in vitro* and *in vivo* results gives the most accurate representation of fetal drug toxicity. Adapting these models to mimic human development will enable the prediction of drug targets, and thus the effect on embryogenesis, in the preclinical phase of drug development. Ultimately, these will give the best indication of a given agents potential teratogenic properties, allowing physicians and patients to make a well-informed decision on their treatment course.

Acknowledgements

Shaunna Beedie is a recipient of a Wellcome Trust-NIH PhD Studentship (Grant number 098252/Z/12/Z). Alexandra J. Diamond is a recipient of a BBSRC EastBIO DTP PhD Scholarship. Lucas Rosa Fraga is a recepient of a PhD scholarship from the Science Without Borders program – CNPq Brazil – INAGEMP/ Grant CNPq 573993/2008-4

Shaunna Beedie and William D. Figg are supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

References

[1] M. Papetti, I.M. Herman, Mechanisms of normal and tumor-derived angiogenesis, Am J Physiol Cell Physiol 282(5) (2002) C947-70.

[2] A. Nakano, H. Nakano, K.A. Smith, N.J. Palpant, The developmental origins and lineage contributions of endocardial endothelium, Biochim Biophys Acta 1863(7 Pt B) (2016) 1937-47.
[3] I.V. Larina, M.D. Garcia, T.J. Vadakkan, K.V. Larin, M.E. Dickinson, Imaging mouse embryonic cardiovascular development, Cold Spring Harb Protoc 2012(10) (2012) 1035-43.
[4] A. Nasevicius, J. Larson, S.C. Ekker, Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant, Yeast 17(4) (2000) 294-301.
[5] E. Ristori, S. Donnini, M. Ziche, Studying Vascular Angiogenesis and Senescence in Zebrafish Embryos, Methods Mol Biol 1430 (2016) 387-400.
[6] H.M. Fraser, S.E. Dickson, K.D. Morris, G.F. Erickson, S.F. Lunn, The effect of the angiogenesis inhibitor TNP-470 on luteal establishment and function in the primate, Hum Reprod 14(8) (1999) 2054-60.
[7] K.P. Chennazhi, N.R. Nayak, Regulation of angiogenesis in the primate endometrium: vascular endothelial growth factor, Semin Reprod Med 27(1) (2009) 80-9.
[8] N. Vargesson, Vascularization of the developing chick limb bud: role of the TGFbeta signalling pathway, J Anat 202(1) (2003) 93-103.
[9] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407(6801) (2000) 249-57.
[10] C.N. Qian, M.H. Tan, J.P. Yang, Y. Cao, Revisiting tumor angiogenesis: vessel co-option, vessel remodeling, and cancer cell-derived vasculature formation, Chin J Cancer 35 (2016) 10.
[11] W.P. Leenders, B. Kusters, R.M. de Waal, Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis, Endothelium 9(2) (2002) 83-7.
[12] L. Coultas, K. Chawengsaksophak, J. Rossant, Endothelial cells and VEGF in vascular development, Nature 438(7070) (2005) 937-45.
[13] S.H. Lee, D. Jeong, Y.S. Han, M.J. Baek, Pivotal role of vascular endothelial growth factor pathway in tumor angiogenesis, Ann Surg Treat Res 89(1) (2015) 1-8.
[14] M.H. Pourgholami, D.L. Morris, Inhibitors of vascular endothelial growth factor in cancer, Cardiovasc Hematol Agents Med Chem 6(4) (2008) 343-7.
[15] T.A. Baudino, C. McKay, H. Pendeville-Samain, J.A. Nilsson, K.H. Maclean, E.L. White, A.C. Davis, J.N. Ihle, J.L. Cleveland, c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression, Gene Dev 16(19) (2002) 2530-2543.
[16] P. Carmeliet, N. Mackman, L. Moons, T. Luther, P. Gressens, I. Van Vlaenderen, H. Demunck, M. Kasper, G. Breier, P. Evrard, M. Muller, W. Risau, T. Edgington, D. Collen, Role of tissue factor in embryonic blood vessel development, Nature 383(6595) (1996) 73-5.
[17] T.B. Knudsen, N.C. Kleinstreuer, Disruption of embryonic vascular development in predictive toxicology, Birth Defects Res C Embryo Today 93(4) (2011) 312-23.
[18] P. Carmeliet, V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq,

J. Pawling, L. Moons, D. Collen, W. Risau, A. Nagy, Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele, Nature 380(6573) (1996) 435-439.
[19] D. Vittet, T. Buchou, A. Schweitzer, E. Dejana, P. Huber, Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies, Proceedings of the National Academy of Sciences of the United States of America 94(12) (1997) 6273-6278.
[20] L.E. Kotch, N.V. Iyer, E. Laughner, G.L. Semenza, Defective vascularization of HIF-1 alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death, Developmental Biology 209(2) (1999) 254-267.
[21] T. Kitsukawa, A. Shimono, A. Kawakami, H. Kondoh, H. Fujisawa, Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs, Development 121(12) (1995) 4309-4318.
[22] A.K. Greene, G.F. Rogers, J.B. Mulliken, Schimmelpenning syndrome: an association with vascular anomalies, Cleft Palate Craniofac J 44(2) (2007) 208-15.
[23] M.I. Van Allen, Structural anomalies resulting from vascular disruption, Pediatr Clin North Am 39(2) (1992) 255-77.
[24] N. Vargesson, D.R. Hootnick, Arterial dysgenesis and limb defects: Clinical and experimental examples, Reproductive Toxicology (In Press).
[25] M.U. Saeed, E. Gkaragkani, K. Ali, Emerging roles for antiangiogenesis factors in management of ocular disease, Clin Ophthalmol 6 (2013) 533-43.
[26] A.N. Witmer, G.F. Vrensen, C.J. Van Noorden, R.O. Schlingemann, Vascular endothelial growth factors and angiogenesis in eye disease, Prog Retin Eye Res 22(1) (2003) 1-29.
[27] S. Danese, M. Sans, C. de la Motte, C. Graziani, G. West, M.H. Phillips, R. Pola, S. Rutella, J. Willis, A. Gasbarrini, C. Fiocchi, Angiogenesis as a novel component of inflammatory bowel disease pathogenesis, Gastroenterology 130(7) (2006) 2060-73.
[28] J.B. Marriott, G. Muller, A.G. Dalgleish, Thalidomide as an emerging immunotherapeutic agent, Immunol Today 20(12) (1999) 538-40.
[29] K.J. Silverman, D.P. Lund, B.R. Zetter, L.L. Lainey, J.A. Shahood, D.G. Freiman, J. Folkman, A.C. Barger, Angiogenic activity of adipose tissue, Biochem Biophys Res Commun 153(1) (1988) 347-52.
[30] M.W. Laschke, M.D. Menger, Anti-angiogenic treatment strategies for the therapy of endometriosis, Hum Reprod Update 18(6) (2012) 682-702.
[31] A.W. Nap, A.W. Griffioen, G.A. Dunselman, J.C. Bouma-Ter Steege, V.L. Thijssen, J.L. Evers, P.G. Groothuis, Antiangiogenesis therapy for endometriosis, J Clin Endocrinol Metab 89(3) (2004) 1089-95.
[32] A.L. Rocha, F.M. Reis, R.N. Taylor, Angiogenesis and endometriosis, Obstet Gynecol Int 2013 (2013) 859619.
[33] T. Takagi, C. van Bennekom, S. Amann, C. Hattori, Y. Shirakuni, T. Sato, M. Nasu, [Risk Management of Teratogenic Drugs ~The Current States of Practice in Europe, US and Japan~], Yakugaku Zasshi 135(10) (2015) 1161-8.

[34] H. Nau, Species-Differences in Pharmacokinetics and Drug Teratogenesis, Environ Health Persp 70 (1986) 113-129.
[35] M.E. Franks, G.R. Macpherson, W.D. Figg, Thalidomide, Lancet 363(9423) (2004) 1802-11.
[36] N. Vargesson, Thalidomide Embryopathy: An enigmatic challenge, ISRN Developmental Biology (Article ID 241016) (2013) http://dx.doi.org/10.1155/2013/241016.
[37] N. Vargesson, Thalidomide-induced teratogenesis: history and mechanisms, Birth Defects Res C Embryo Today 105(2) (2015) 140-56.
[38] F.O. Kelsey, Thalidomide update: regulatory aspects, Teratology 38(3) (1988) 221-6.
[39] C. Pardo-Martin, T.Y. Chang, B.K. Koo, C.L. Gilleland, S.C. Wasserman, M.F. Yanik, High-throughput in vivo vertebrate screening, Nat Methods 7(8) (2010) 634-6.
[40] M.N. Chavez, G. Aedo, F.A. Fierro, M.L. Allende, J.T. Egana, Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration, Front Physiol 7 (2016) 56.
[41] G.N. Serbedzija, E. Flynn, C.E. Willett, Zebrafish angiogenesis: a new model for drug screening, Angiogenesis 3(4) (1999) 353-9.
[42] S. Zhao, J. Huang, J. Ye, A fresh look at zebrafish from the perspective of cancer research, J Exp Clin Canc Res 34 (2015).
[43] A. Schuermann, C.S. Helker, W. Herzog, Angiogenesis in zebrafish, Semin Cell Dev Biol 31 (2014) 106-14.
[44] D. Liang, J.R. Chang, A.J. Chin, A. Smith, C. Kelly, E.S. Weinberg, R. Ge, The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development, Mech Dev 108(1-2) (2001) 29-43.
[45] L.D. Jensen, P. Rouhi, Z. Cao, T. Lanne, E. Wahlberg, Y. Cao, Zebrafish models to study hypoxia-induced pathological angiogenesis in malignant and nonmalignant diseases, Birth Defects Res C Embryo Today 93(2) (2011) 182-93.
[46] S. Childs, J.N. Chen, D.M. Garrity, M.C. Fishman, Patterning of angiogenesis in the zebrafish embryo, Development 129(4) (2002) 973-82.
[47] S. Rezzola, G. Paganini, F. Semeraro, M. Presta, C. Tobia, Zebrafish (Danio rerio) embryo as a platform for the identification of novel angiogenesis inhibitors of retinal vascular diseases, Biochim Biophys Acta 1862(7) (2016) 1291-6.
[48] X. Qi, G. Liu, L. Qiu, X. Lin, M. Liu, Marine bromophenol bis(2,3-dibromo-4,5-dihydroxybenzyl) ether, represses angiogenesis in HUVEC cells and in zebrafish embryos via inhibiting the VEGF signal systems, Biomed Pharmacother 75 (2015) 58-66.
[49] J. Cheng, Y.J. Gu, Y. Wang, S.H. Cheng, W.T. Wong, Nanotherapeutics in angiogenesis: synthesis and in vivo assessment of drug efficacy and biocompatibility in zebrafish embryos, Int J Nanomedicine 6 (2011) 2007-21.
[50] S.L. Beedie, C.J. Peer, S. Pisle, E.R. Gardner, C. Mahony, S. Barnett, A. Ambrozak, M. Gutschow, C.H. Chau, N. Vargesson, W.D. Figg, Anticancer Properties of a Novel Class of Tetrafluorinated Thalidomide Analogues, Molecular Cancer Therapeutics 14(10) (2015) 2228-37.

[51] S.L. Beedie, H.M. Rore, S. Barnett, C.H. Chau, W. Luo, N.H. Greig, W.D. Figg, N. Vargesson, In vivo screening and discovery of novel candidate thalidomide analogs in the zebrafish embryo and chicken embryo model systems, Oncotarget 7(22) (2016) 33237-45.
[52] C. Mahony, L. Erskine, J. Niven, N.H. Greig, W.D. Figg, N. Vargesson, Pomalidomide is nonteratogenic in chicken and zebrafish embryos and nonneurotoxic in vitro, Proceedings of the National Academy of Sciences of the United States of America 110(31) (2013) 12703-8.
[53] A. Papakyriakou, P. Kefalos, P. Sarantis, C. Tsiamantas, K.P. Xanthopoulos, D. Vourloumis, D. Beis, A zebrafish in vivo phenotypic assay to identify 3-aminothiophene-2-carboxylic acid-based angiogenesis inhibitors, Assay Drug Dev Technol 12(9-10) (2014) 527-35.
[54] S.L. Beedie, C. Mahony, H.M. Walker, C.H. Chau, W.D. Figg, N. Vargesson, Shared mechanism of teratogenicity of anti-angiogenic drugs identified in the chicken embryo model, Sci Rep 6 (2016) 30038.
[55] E. Ellertsdottir, A. Lenard, Y. Blum, A. Krudewig, L. Herwig, M. Affolter, H.G. Belting, Vascular morphogenesis in the zebrafish embryo, Dev Biol 341(1) (2010) 56-65.
[56] J. Bussmann, N. Lawson, L. Zon, S. Schulte-Merker, C. Zebrafish Nomenclature, Zebrafish VEGF receptors: a guideline to nomenclature, PLoS Genet 4(5) (2008) e1000064.
[57] J.E. Cannon, P.D. Upton, J.C. Smith, N.W. Morrell, Intersegmental vessel formation in zebrafish: requirement for VEGF but not BMP signalling revealed by selective and non-selective BMP antagonists, Br J Pharmacol 161(1) (2010) 140-9.
[58] J.D. Larson, S.A. Wadman, E. Chen, L. Kerley, K.J. Clark, M. Eide, S. Lippert, A. Nasevicius, S.C. Ekker, P.B. Hackett, J.J. Essner, Expression of VE-cadherin in zebrafish embryos: a new tool to evaluate vascular development, Developmental dynamics : an official publication of the American Association of Anatomists 231(1) (2004) 204-13.
[59] M.M. Santoro, Antiangiogenic cancer drug using the zebrafish model, Arterioscler Thromb Vasc Biol 34(9) (2014) 1846-53.
[60] C. Tobia, G. Gariano, J. Guerra, M. Presta, Zebrafish embryo intersegmental vessels: a tool for investigating sprouting angiogenesis, Methods Mol Biol 1214 (2015) 173-84.
[61] N.D. Lawson, B.M. Weinstein, In vivo imaging of embryonic vascular development using transgenic zebrafish, Dev Biol 248(2) (2002) 307-18.
[62] G. Chimote, J. Sreenivasan, N. Pawar, J. Subramanian, H. Sivaramakrishnan, S. Sharma, Comparison of effects of anti-angiogenic agents in the zebrafish efficacy-toxicity model for translational anti-angiogenic drug discovery, Drug Des Devel Ther 8 (2014) 1107-23.
[63] C.A. Staton, S.M. Stribbling, S. Tazzyman, R. Hughes, N.J. Brown, C.E. Lewis, Current methods for assaying angiogenesis in vitro and in vivo, Int J Exp Pathol 85(5) (2004) 233-48.
[64] C. Therapontos, L. Erskine, E.R. Gardner, W.D. Figg, N. Vargesson, Thalidomide induces limb defects by preventing angiogenic outgrowth during

early limb formation, Proceedings of the National Academy of Sciences of the United States of America 106(21) (2009) 8573-8.
[65] A.L. Reynolds, Y. Alvarez, T. Sasore, N. Waghorne, C.T. Butler, C. Kilty, A.J. Smith, C. McVicar, V.H. Wong, O. Galvin, S. Merrigan, J. Osman, G. Grebnev, A. Sjolander, A.W. Stitt, B.N. Kennedy, Phenotype-based Discovery of 2-[(E)-2-(Quinolin-2-yl)vinyl]phenol as a Novel Regulator of Ocular Angiogenesis, J Biol Chem 291(14) (2016) 7242-55.
[66] T. Sasore, B. Kennedy, Deciphering combinations of PI3K/AKT/mTOR pathway drugs augmenting anti-angiogenic efficacy in vivo, PloS one 9(8) (2014) e105280.
[67] J. Chan, P.E. Bayliss, J.M. Wood, T.M. Roberts, Dissection of angiogenic signaling in zebrafish using a chemical genetic approach, Cancer Cell 1(3) (2002) 257-67.
[68] G. Wang, S.K. Rajpurohit, F. Delaspre, S.L. Walker, D.T. White, A. Ceasrine, R. Kuruvilla, R.J. Li, J.S. Shim, J.O. Liu, M.J. Parsons, J.S. Mumm, First quantitative high-throughput screen in zebrafish identifies novel pathways for increasing pancreatic beta-cell mass, Elife 4 (2015).
[69] S. Nicoli, M. Presta, The zebrafish/tumor xenograft angiogenesis assay, Nature Protocols 2(11) (2007) 2918-2923.
[70] T. Yabu, H. Tomimoto, Y. Taguchi, S. Yamaoka, Y. Igarashi, T. Okazaki, Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate, Blood 106(1) (2005) 125-34.
[71] T. Ito, H. Ando, T. Suzuki, T. Ogura, K. Hotta, Y. Imamura, Y. Yamaguchi, H. Handa, Identification of a primary target of thalidomide teratogenicity, Science 327(5971) (2010) 1345-50.
[72] A. Broyl, R. Kuiper, M. van Duin, B. van der Holt, L. el Jarari, U. Bertsch, S. Zweegman, A. Buijs, D. Hose, H.M. Lokhorst, H. Goldschmidt, P. Sonneveld, H.g. Dutch-Belgian, G.G. German, High cereblon expression is associated with better survival in patients with newly diagnosed multiple myeloma treated with thalidomide maintenance, Blood 121(4) (2013) 624-7.
[73] Y.X. Zhu, E. Braggio, C.X. Shi, L.A. Bruins, J.E. Schmidt, S. Van Wier, X.B. Chang, C.C. Bjorklund, R. Fonseca, P.L. Bergsagel, R.Z. Orlowski, A.K. Stewart, Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide, Blood 118(18) (2011) 4771-9.
[74] C.C. Bjorklund, L. Lu, J. Kang, P.R. Hagner, C.G. Havens, M. Amatangelo, M. Wang, Y. Ren, S. Couto, M. Breider, Y. Ning, A.K. Gandhi, T.O. Daniel, R. Chopra, A. Klippel, A.G. Thakurta, Rate of CRL4(CRBN) substrate Ikaros and Aiolos degradation underlies differential activity of lenalidomide and pomalidomide in multiple myeloma cells by regulation of c-Myc and IRF4, Blood Cancer J 5 (2015) e354.
[75] E. Iskierka-Jazdzewska, A. Stepien, F. Canzian, A. Martino, D. Campa, A. Stein, M. Krawczyk-Kulis, M. Rybicka, S. Kyrcz-Krzemien, A.K. Butrym, G. Mazur, A.J. Jurczyszyn, D. Zawirska, N. Grzasko, W. Tomczak, E. Subocz, M. Watek, M. Pasiarski, M. Rymko, M. Calbecka, A. Druzd-Sitek, J. Walewski, M. Kruszewski, M. Razny, J.M. Zaucha, M. Dudzinski, P. Gaj, K. Warzocha, K.

Jamroziak, Cereblon (CRBN) Gene Polymorphisms Predict Clinical Response and Progression-Free Survival in Multiple Myeloma Patients Treated with Lenalidomide: A Pharmacogenetic Study of Immense Consortium, Blood 124(21) (2014).
[76] M.G. Davey, C. Tickle, The chicken as a model for embryonic development, Cytogenet Genome Res 117(1-4) (2007) 231-9.
[77] C. Tabin, L. Wolpert, Rethinking the proximodistal axis of the vertebrate limb in the molecular era, Genes Dev 21(12) (2007) 1433-42.
[78] N. Vargesson, E. Laufer, Smad7 misexpression during embryonic angiogenesis causes vascular dilation and malformations independently of vascular smooth muscle cell function, Dev Biol 240(2) (2001) 499-516.
[79] C. Mahony, N. Vargesson, Molecular analysis of regulative events in the developing chick limb, J Anat 223(1) (2013) 1-13.
[80] S. Bjornstad, L.P. Austdal, B. Roald, J.C. Glover, R.E. Paulsen, Cracking the Egg: Potential of the Developing Chicken as a Model System for Nonclinical Safety Studies of Pharmaceuticals, J Pharmacol Exp Ther 355(3) (2015) 386-96.
[81] A. Martowicz, J. Kern, E. Gunsilius, G. Untergasser, Establishment of a human multiple myeloma xenograft model in the chicken to study tumor growth, invasion and angiogenesis, J Vis Exp (99) (2015) e52665.
[82] M. Moriyama, S. Metzger, A.J. van der Vlies, H. Uyama, M. Ehrbar, U. Hasegawa, Inhibition of angiogenesis by antioxidant micelles, Adv Healthc Mater 4(4) (2015) 569-75.
[83] P. Nowak-Sliwinska, T. Segura, M.L. Iruela-Arispe, The chicken chorioallantoic membrane model in biology, medicine and bioengineering, Angiogenesis 17(4) (2014) 779-804.
[84] A.I. Whitsel, C.B. Johnson, C.J. Forehand, An in ovo chicken model to study the systemic and localized teratogenic effects of valproic acid, Teratology 66(4) (2002) 153-163.
[85] L. Akhtar, M.Y. Khan, L.A. Minhas, The effect of prenatal administration of valproic acid on the survivability and day of hatching of chick embryo, Journal of the Pakistan Medical Association 65(2) (2015) 175-178.
[86] L. Cincarova, Z. Zdrahal, J. Fajkus, New perspectives of valproic acid in clinical practice, Expert Opin Inv Drug 22(12) (2013) 1535-1547.
[87] M. Tanoshima, T. Kobayashi, R. Tanoshima, J. Beyene, G. Koren, S. Ito, Risks of Congenital Malformations in Offspring Exposed to Valproic Acid In Utero: A Systematic Review and Cumulative Meta-analysis, Clinical Pharmacology & Therapeutics 98(4) (2015) 417-441.
[88] R.M. Nanau, M.G. Neuman, Adverse drug reactions induced by valproic acid, Clinical Biochemistry 46(15) (2013) 1323-1338.
[89] P.G. Kelly, C.M. Regan, Studies on valproate-induced perturbations of neurulation in the explanted chick embryo, Toxicology 71(1-2) (1992) 137-44.
[90] C. Wegner, H. Nau, Alteration of embryonic folate metabolism by valproic acid during organogenesis: implications for mechanism of teratogenesis, Neurology 42(4 Suppl 5) (1992) 17-24.

[91] F. Azarbayjani, B.R. Danielsson, Pharmacologically induced embryonic dysrhythmia and episodes of hypoxia followed by reoxygenation: a common teratogenic mechanism for antiepileptic drugs?, Teratology 57(3) (1998) 117-26.
[92] G. Fex, K. Larsson, A. Andersson, M. Berggren-Soderlund, Low serum concentration of all-trans and 13-cis retinoic acids in patients treated with phenytoin, carbamazepine and valproate. Possible relation to teratogenicity, Arch Toxicol 69(8) (1995) 572-4.
[93] H. Nau, G. Tzimas, M. Mondry, C. Plum, H.L. Spohr, Antiepileptic drugs alter endogenous retinoid concentrations: a possible mechanism of teratogenesis of anticonvulsant therapy, Life Sci 57(1) (1995) 53-60.
[94] J.B. Boylen, H.H. Horne, W.J. Johnson, Teratogenic effects of thalidomide and related substances, Lancet 1(7280) (1963) 552.
[95] T.D. Stephens, The effect of thalidomide in chicken embryos, Birth Defects Res A Clin Mol Teratol 85(8) (2009) 725-31.
[96] A. Jurand, Early changes in limb buds of chick embryos after thalidomide treatment, J Embryol Exp Morphol 16(2) (1966) 289-300.
[97] J. Knobloch, J.D. Shaughnessy, Jr., U. Ruther, Thalidomide induces limb deformities by perturbing the Bmp/Dkk1/Wnt signaling pathway, FASEB J 21(7) (2007) 1410-21.
[98] N. Vargesson, Thalidomide-induced limb defects: resolving a 50-year-old puzzle, Bioessays 31(12) (2009) 1327-36.
[99] K.P. Tamilarasan, G.K. Kolluru, M. Rajaram, M. Indhumathy, R. Saranya, S. Chatterjee, Thalidomide attenuates nitric oxide mediated angiogenesis by blocking migration of endothelial cells, BMC Cell Biol 7 (2006) 17.
[100] J. Knobloch, D. Jungck, A. Koch, Apoptosis induction by thalidomide: critical for limb teratogenicity but therapeutic potential in idiopathic pulmonary fibrosis?, Curr Mol Pharmacol 4(1) (2011) 26-61.
[101] K.T. Al-Jamal, W.T. Al-Jamal, S. Akerman, J.E. Podesta, A. Yilmazer, J.A. Turton, A. Bianco, N. Vargesson, C. Kanthou, A.T. Florence, G.M. Tozer, K. Kostarelos, Systemic antiangiogenic activity of cationic poly-L-lysine dendrimer delays tumor growth, Proceedings of the National Academy of Sciences of the United States of America 107(9) (2010) 3966-3971.
[102] D. Ribatti, The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model, Mech Dev 141 (2016) 70-77.
[103] R.H. Waterston, K. Lindblad-Toh, E. Birney, J. Rogers, J.F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S.E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M.R. Brent, D.G. Brown, S.D. Brown, C. Bult, J. Burton, J. Butler, R.D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A.T. Chinwalla, D.M. Church, M. Clamp, C. Clee, F.S. Collins, L.L. Cook, R.R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K.D. Delehaunty, J. Deri, E.T. Dermitzakis, C. Dewey, N.J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D.M. Dunn, S.R. Eddy, L. Elnitski, R.D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G.A. Fewell, P. Flicek, K. Foley, W.N. Frankel, L.A. Fulton, R.S. Fulton, T.S. Furey, D. Gage, R.A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T.A. Graves, E.D.

Green, S. Gregory, R. Guigo, M. Guyer, R.C. Hardison, D. Haussler, Y. Hayashizaki, L.W. Hillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D.B. Jaffe, L.S. Johnson, M. Jones, T.A. Jones, A. Joy, M. Kamal, E.K. Karlsson, D. Karolchik, A. Kasprzyk, J. Kawai, E. Keibler, C. Kells, W.J. Kent, A. Kirby, D.L. Kolbe, I. Korf, R.S. Kucherlapati, E.J. Kulbokas, D. Kulp, T. Landers, J.P. Leger, S. Leonard, I. Letunic, R. Levine, J. Li, M. Li, C. Lloyd, S. Lucas, B. Ma, D.R. Maglott, E.R. Mardis, L. Matthews, E. Mauceli, J.H. Mayer, M. McCarthy, W.R. McCombie, S. McLaren, K. McLay, J.D. McPherson, J. Meldrim, B. Meredith, J.P. Mesirov, W. Miller, T.L. Miner, E. Mongin, K.T. Montgomery, M. Morgan, R. Mott, J.C. Mullikin, D.M. Muzny, W.E. Nash, J.O. Nelson, M.N. Nhan, R. Nicol, Z. Ning, C. Nusbaum, M.J. O'Connor, Y. Okazaki, K. Oliver, E.O. Larty, L. Pachter, G. Parra, K.H. Pepin, J. Peterson, P. Pevzner, R. Plumb, C.S. Pohl, A. Poliakov, T.C. Ponce, C.P. Ponting, S. Potter, M. Quail, A. Reymond, B.A. Roe, K.M. Roskin, E.M. Rubin, A.G. Rust, R. Santos, V. Sapojnikov, B. Schultz, J. Schultz, M.S. Schwartz, S. Schwartz, C. Scott, S. Seaman, S. Searle, T. Sharpe, A. Sheridan, R. Shownkeen, S. Sims, J.B. Singer, G. Slater, A. Smit, D.R. Smith, B. Spencer, A. Stabenau, N.S. Strange-Thomann, C. Sugnet, M. Suyama, G. Tesler, J. Thompson, D. Torrents, E. Trevaskis, J. Tromp, C. Ucla, A.U. Vidal, J.P. Vinson, A.C. von Niederhausern, C.M. Wade, M. Wall, R.J. Weber, R.B. Weiss, M.C. Wendl, A.P. West, K. Wetterstrand, R. Wheeler, S. Whelan, J. Wierzbowski, D. Willey, S. Williams, R.K. Wilson, E. Winter, K.C. Worley, D. Wyman, S. Yang, S.P. Yang, E.M. Zdobnov, M.C. Zody, E.S. Lander, M.G.S. Consor, Initial sequencing and comparative analysis of the mouse genome, Nature 420(6915) (2002) 520-562.
[104] D. Nguyen, T. Xu, The expanding role of mouse genetics for understanding human biology and disease, Disease Models & Mechanisms 1(1) (2008) 56-66.
[105] W. Risau, Mechanisms of angiogenesis, Nature 386(6626) (1997) 671-674.
[106] C.J. Drake, P.A. Fleming, Vasculogenesis in the day 6.5 to 9.5 mouse embryo, Blood 95(5) (2000) 1671-1679.
[107] J. Palis, M.C. Yoder, Yolk-sac hematopoiesis: The first blood cells of mouse and man, Experimental Hematology 29(8) (2001) 927-936.
[108] J. Palis, S. Robertson, M. Kennedy, C. Wall, G. Keller, Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse, Development 126(22) (1999) 5073-5084.
[109] K.J. Kim, B. Li, J. Winer, M. Armanini, N. Gillett, H.S. Phillips, N. Ferrara, Inhibition of Vascular Endothelial Growth Factor-Induced Angiogenesis Suppresses Tumor-Growth Invivo, Nature 362(6423) (1993) 841-844.
[110] A.P. Adamis, J.W. Miller, M.T. Bernal, D.J. Damico, J. Folkman, T.K. Yeo, K.T. Yeo, Increased Vascular Endothelial Growth-Factor Levels in the Vitreous of Eyes with Proliferative Diabetic-Retinopathy, American Journal of Ophthalmology 118(4) (1994) 445-450.
[111] L.P. Aiello, R.L. Avery, P.G. Arrigg, B.A. Keyt, H.D. Jampel, S.T. Shah, L.R. Pasquale, H. Thieme, M.A. Iwamoto, J.E. Park, H.V. Nguyen, L.M. Aiello, N. Ferrara, G.L. King, Vascular Endothelial Growth-Factor in Ocular Fluid of Patients with Diabetic-Retinopathy and Other Retinal Disorders, New Engl J Med 331(22) (1994) 1480-1487.

[112] T.N. Sato, Y. Tozawa, U. Deutsch, K. Wolburgbuchholz, Y. Fujiwara, M. Gendronmaguire, T. Gridley, H. Wolburg, W. Risau, Y. Qin, Distinct Roles of the Receptor Tyrosine Kinases Tie-1 and Tie-2 in Blood-Vessel Formation, Nature 376(6535) (1995) 70-74.
[113] C. Suri, P.F. Jones, S. Patan, S. Bartunkova, P.C. Maisonpierre, S. Davis, T.N. Sato, G.D. Yancopoulos, Requisite role of Angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis, Cell 87(7) (1996) 1171-1180.
[114] J. Folkman, E. Merler, Abernath.C, G. Williams, Isolation of a Tumor Factor Responsible for Angiogenesis, Journal of Experimental Medicine 133(2) (1971) 275-288.
[115] J. Folkman, C.C. Haudenschild, B.R. Zetter, Long-Term Culture of Capillary Endothelial-Cells, Proceedings of the National Academy of Sciences of the United States of America 76(10) (1979) 5217-5221.
[116] L. Eklund, M. Bry, K. Alitalo, Mouse models for studying angiogenesis and lymphangiogenesis in cancer, Mol Oncol 7(2) (2013) 259-282.
[117] T. Couffinhal, M. Silver, L.P. Zheng, M. Kearney, B. Witzenbichler, J.M. Isner, Mouse model of angiogenesis, American Journal of Pathology 152(6) (1998) 1667-1679.
[118] J.P.M. Cleutjens, W.M. Blankesteijn, M.J.A.P. Daemen, J.F.M. Smits, The infarcted myocardium: Simply dead tissue, or a lively target for therapeutic interventions, Cardiovascular Research 44(2) (1999) 232-241.
[119] G.H. Fong, J. Rossant, M. Gertsenstein, M.L. Breitman, Role of the Flt-1 Receptor Tyrosine Kinase in Regulating the Assembly of Vascular Endothelium, Nature 376(6535) (1995) 66-70.
[120] M.C. Puri, J. Rossant, K. Alitalo, A. Bernstein, J. Partanen, The Receptor Tyrosine Kinase Tie Is Required for Integrity and Survival of Vascular Endothelial-Cells, Embo Journal 14(23) (1995) 5884-5891.
[121] F. Shalaby, J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman, A.C. Schuh, Failure of Blood-Island Formation and Vasculogenesis in Flk-1-Deficient Mice, Nature 376(6535) (1995) 62-66.
[122] P.C. Maisonpierre, C. Suri, P.F. Jones, S. Bartunkova, S. Wiegand, C. Radziejewski, D. Compton, J. McClain, T.H. Aldrich, N. Papadopoulos, T.J. Daly, S. Davis, T.N. Sato, G.D. Yancopoulos, Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis, Science 277(5322) (1997) 55-60.
[123] V.C. Ho, L.J. Duan, C. Cronin, B.T. Liang, G.H. Fong, Elevated vascular endothelial growth factor receptor-2 abundance contributes to increased angiogenesis in vascular endothelial growth factor receptor-1-deficient mice, Circulation 126(6) (2012) 741-52.
[124] J. Andrae, R. Gallini, C. Betsholtz, Role of platelet-derived growth factors in physiology and medicine, Genes Dev 22(10) (2008) 1276-312.
[125] R.H. Adams, A. Eichmann, Axon guidance molecules in vascular patterning, Cold Spring Harb Perspect Biol 2(5) (2010) a001875.
[126] M. Jeltsch, V.M. Leppanen, P. Saharinen, K. Alitalo, Receptor tyrosine kinase-mediated angiogenesis, Cold Spring Harb Perspect Biol 5(9) (2013).

[127] Y.H. Cao, Antiangiogenic cancer therapy: why do mouse and human patients respond in a different way to the same drug?, International Journal of Developmental Biology 55(4-5) (2011) 557-562.
[128] I.D. Fratta, E.B. Sigg, K. Maiorana, Teratogenic Effects of Thalidomide in Rabbits Rats Hamsters and Mice, Toxicol Appl Pharm 7(2) (1965) 268-268.
[129] E.O. Hagen, Y. Tzuszu, Drugs and Congenital Abnormalities, Lancet 1(727) (1963) 501-501.
[130] V. Knapp, G.A. Christie, M.J. Seller, Thalidomide and Congenital Abnormalities, Lancet 2(7249) (1962) 249-249.
[131] J.A. Dipaolo, J. Pickren, H. Gatzek, Malformations Induced in Mouse by Thalidomide, Anat Rec 149(1) (1964) 149-149.
[132] C. Petter, Early foetal thrombosis induced by thalidomide in mouse: possible explanation for teratogenicity, Experientia 33(10) (1977) 1384-6.
[133] K.L. Hallene, E. Oby, B.J. Lee, S. Santaguida, S. Bassanini, M. Cipolla, N. Marchi, M. Hossain, G. Battaglia, D. Janigro, Prenatal exposure to thalidomide, altered vasculogenesis, and CNS malformations, Neuroscience 142(1) (2006) 267-283.
[134] F. Chung, J. Lu, B.D. Palmer, P. Kestell, P. Browett, B.C. Baguley, M. Tingle, L.M. Ching, Thalidomide pharmacokinetics and metabolite formation in mice, rabbits, and multiple myeloma patients, Clinical Cancer Research 10(17) (2004) 5949-5956.
[135] J. Knobloch, I. Schmitz, K. Gotz, K. Schulze-Osthoff, U. Ruther, Thalidomide induces limb anomalies by PTEN stabilization, Akt suppression, and stimulation of caspase-dependent cell death, Molecular and Cellular Biology 28(2) (2008) 529-538.
[136] T.D. Stephens, C.J. Bunde, B.J. Fillmore, Mechanism of action in thalidomide teratogenesis, Biochem Pharmacol 59(12) (2000) 1489-99.
[137] K.S. Bauer, S.C. Dixon, W.D. Figg, Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent, Biochem Pharmacol 55(11) (1998) 1827-34.
[138] Y. Ando, E. Fuse, W.D. Figg, Thalidomide metabolism by the CYP2C subfamily, Clin Cancer Res 8(6) (2002) 1964-73.
[139] E.R. Lepper, N.F. Smith, M.C. Cox, C.D. Scripture, W.D. Figg, Thalidomide metabolism and hydrolysis: mechanisms and implications, Curr Drug Metab 7(6) (2006) 677-85.
[140] Y. Kazuki, M. Akita, K. Kobayashi, M. Osaki, D. Satoh, R. Ohta, S. Abe, S. Takehara, K. Kazuki, H. Yamazaki, T. Kamataki, M. Oshimura, Thalidomide-induced limb abnormalities in a humanized CYP3A mouse model, Sci Rep 6 (2016) 21419.
[141] M. Saint-Geniez, A.S.R. Maharaj, T.E. Walshe, B.A. Tucker, E. Sekiyama, T. Kurihara, D.C. Darland, M.J. Young, P.A. D'Amore, Endogenous VEGF Is Required for Visual Function: Evidence for a Survival Role on Muller Cells and Photoreceptors, PloS one 3(11) (2008).
[142] M.S. Oreilly, L. Holmgren, Y. Shing, C. Chen, R.A. Rosenthal, M. Moses, W.S. Lane, Y.H. Cao, E.H. Sage, J. Folkman, Angiostatin - a Novel Angiogenesis

Inhibitor That Mediates the Suppression of Metastases by a Lewis Lung-Carcinoma, Cell 79(2) (1994) 315-328.
[143] B. Fischer, P. Chavatte-Palmer, C. Viebahn, A.N. Santos, V. Duranthon, Rabbit as a reproductive model for human health, Reproduction 144(1) (2012) 1-10.
[144] R.H. Foote, E.W. Carney, The rabbit as a model for reproductive and developmental toxicity studies, Reproductive Toxicology 14(6) (2000) 477-493.
[145] G. Janer, W. Slob, B.C. Hakkert, T. Vermeire, A.H. Piersma, A retrospective analysis of developmental toxicity studies in rat and rabbit: What is the added value of the rabbit as an additional test species?, Regul Toxicol Pharm 50(2) (2008) 206-217.
[146] C.J.J. Lee, L.L. Goncalves, P.G. Wells, Embryopathic effects of thalidomide and its hydrolysis products in rabbit embryo culture: evidence for a prostaglandin H synthase (PHS)-dependent, reactive oxygen species (ROS)-mediated mechanism, Faseb Journal 25(7) (2011) 2468-2483.
[147] P.T. Theunissen, S. Beken, B. Beyer, W.J. Breslin, G.D. Cappon, C.L. Chen, G. Chmielewski, L. de Schaepdrijver, B. Enright, J.E. Foreman, W. Harrouk, K.W. Hew, A.M. Hoberman, Y.H. J, T.B. Knudsen, S.B. Laffan, S.L. Makris, M. Martin, M.E. McNerney, C.L. Siezen, D.J. Stanislaus, J. Stewart, K.E. Thompson, B. Tornesi, J.W. Van der Laan, G.F. Weinbauer, S. Wood, A.H. Piersma, Comparison of rat and rabbit embryo–fetal developmental toxicity data for 379 pharmaceuticals: on the nature and severity of developmental effects, Critical Reviews in Toxicology (2016) In Press.
[148] P.T. Theunissen, S. Beken, B. Beyer, W.J. Breslin, G.D. Cappon, C.L. Chen, G. Chmielewski, L. de Schaepdrijver, B. Enright, J.E. Foreman, W. Harrouk, K.W. Hew, A.M. Hoberman, Y.H. J, T.B. Knudsen, S.B. Laffan, S.L. Makris, M. Martin, M.E. McNerney, C.L. Siezen, D.J. Stanislaus, J. Stewart, K.E. Thompson, B. Tornesi, J.W. Van der Laan, G.F. Weinbauer, S. Wood, A.H. Piersma, Comparing rat and rabbit embryo-fetal developmental toxicity data for 379 pharmaceuticals: on systemic dose and developmental effects, Crit Rev Toxicol (2016) In Press.
[149] R.J. D'Amato, M.S. Loughnan, E. Flynn, J. Folkman, Thalidomide is an inhibitor of angiogenesis, Proceedings of the National Academy of Sciences of the United States of America 91(9) (1994) 4082-5.
[150] G.J. Chellman, J.L. Bussiere, N. Makori, P.L. Martin, Y. Ooshima, G.F. Weinbauer, Developmental and reproductive toxicology studies in nonhuman primates, Birth Defects Res B Dev Reprod Toxicol 86(6) (2009) 446-62.
[151] E.D. Albrecht, V.A. Robb, G.J. Pepe, Regulation of placental vascular endothelial growth/permeability factor expression and angiogenesis by estrogen during early baboon pregnancy, J Clin Endocr Metab 89(11) (2004) 5803-5809.
[152] D.E. Poswillo, W.J. Hamilton, D. Sopher, The marmoset as an animal model for teratological research, Nature 239(5373) (1972) 460-2.
[153] K. Ishihara-Hattori, P. Barrow, Review of embryo-fetal developmental toxicity studies performed for recent FDA-approved pharmaceuticals, Reprod Toxicol 64 (2016) 98-104.

[154] M. Ema, R. Ise, H. Kato, S. Oneda, A. Hirose, M. Hirata-Koizumi, A.V. Singh, T.B. Knudsen, T. Ihara, Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide, Reproductive Toxicology 29(1) (2010) 49-56.
[155] W. Heger, S. Klug, H.J. Schmahl, H. Nau, H.J. Merker, D. Neubert, Embryotoxic Effects of Thalidomide Derivatives on the Non-Human Primate Callithrix-Jacchus .3. Teratogenic Potency of the Em-12 Enantiomers, Archives of Toxicology 62(2-3) (1988) 205-208.
[156] H.J. Merker, W. Heger, K. Sames, H. Sturje, D. Neubert, Embryotoxic effects of thalidomide-derivatives in the non-human primate Callithrix jacchus. I. Effects of 3-(1,3-dihydro-1-oxo-2H-isoindol-2-yl)-2,6-dioxopiperidine (EM12) on skeletal development, Arch Toxicol 61(3) (1988) 165-79.
[157] R. Neubert, N. Hinz, R. Thiel, D. Neubert, Down-regulation of adhesion receptors on cells of primate embryos as a probable mechanism of the teratogenic action of thalidomide, Life Sci 58(4) (1996) 295-316.
[158] D. Neubert, W. Heger, H.J. Merker, K. Sames, R. Meister, Embryotoxic effects of thalidomide derivatives in the non-human primate Callithrix jacchus. II. Elucidation of the susceptible period and of the variability of embryonic stages, Arch Toxicol 61(3) (1988) 180-91.
[159] J.M. Hansen, K.K. Harris, M.A. Philbert, C. Harris, Thalidomide modulates nuclear redox status and preferentially depletes glutathione in rabbit limb versus rat limb, J Pharmacol Exp Ther 300(3) (2002) 768-76.
[160] P. ten Dijke, H.M. Arthur, Extracellular control of TGFbeta signalling in vascular development and disease, Nat Rev Mol Cell Biol 8(11) (2007) 857-69.
[161] D.M. Noden, Embryonic origins and assembly of blood vessels, Am Rev Respir Dis 140(4) (1989) 1097-103.
[162] S. Chakrabarti, C.J. Barrow, R.K. Kanwar, V. Ramana, J.R. Kanwar, Current protein-based anti-angiogenic therapeutics, Mini Rev Med Chem 14(3) (2014) 291-312.

Figure Legends

Figure 1: **Vasculogenesis and angiogenesis**. Endothelial cell precursors (angioblasts) are derived from mesodermal stem cells. Angioblasts are promoted by vascular endothelial growth factor (VEGF) and its co-receptor neuropilin-1, to migrate, differentiate, and assemble into endothelial cords. The primitive vascular plexus forms from merging of the endothelial cords with the endocardial tubes (vasculogenesis). The expansion and remodeling (angiogenesis) leads to stabilized vessels in a vascular network. This is mediated by VEGF, as well as other factors (such as Sonic hedgehog and Notch signaling). Vessels are

stabilized by mural cells, a process mediated by platelet derived growth factor B (PDGF) acting upon its receptor (PDGFR). Adapted from [160] and [161].

Figure 2: **Molecular mechanisms controlling angiogenesis**.

Figure 3: **Angiogenesis and angiogenesis inhibitors**. (A) Angiogenesis is promoted by tumour cells and neighbouring stromal cells. Endothelial cells are activated by pro-angiogenic factors released by these cells and they begin to migrate. They then proliferate and stabilize, while the angiogenic factors continue to promote the process in a feedback loop. Vessels penetrate the environment of the cancerous cells allowing the tumour to grow. (B) At the molecular level, angiogenesis inhibitors reduce the inappropriate blood vessel growth by targeting VEGF receptors directly (sunitinib, sorafenib, axitinib), a downstream molecular target (sorafenib, vandetanib, pazopanib) or by acting within the tumour cells themselves to inhibit the production of pro-angiogenic factors such as VEGF (everolimus) [54, 162].

Figure 4: **Angiogenesis in the zebrafish and developing chicken embryo as a tool for assessing drug action**. (A) Normal vascular development at 24 hours post fertilization and (B) reduced vascular development in an anti-angiogenic drug (sunitinib) treated transgenic *fli1*:EGFP embryo. Angiogenesis is quantified by measuring intersomitic vessel (ISV) number and outgrowth. Selected ISVs are indicated by white arrow heads. The anterior (head) and posterior (tail) regions are indicated. The yolk sac (YS) is indicated. (C) A chicken embryo with normal eye (e), limb bud (l) and spinal (s) development. (D) Chicken embryo following treatment for 24 hours with an anti-angiogenic drug (sunitinib). Note hemorrhagic tissues (black asterisk) and bent spine of the embryo (black arrowhead). The chorioallantoic membrane (CAM) and yolk sac membrane (YSM) are indicated. Images are from previously unpublished work of Beedie, Figg and Vargesson from a published study [54].