

Genes and mechanisms that are involved in the effects of teratogens on limb development

And application of alternative models in limb (mal)development research

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Abstract

This review focuses on teratogens that cause limb malformations and the underlying mechanisms they deregulate to cause this. Normal limb development starts with proliferation of the intermediate mesoderm at sites along the body's antero-posterior axis designated to form limbs. This is followed by axis patterning, bone and cartilage formation, muscle formation and vascular development to form a complete limb. Exposure to retinoic acid, alcohol, thalidomide, warfarin and valproic acid during limb development are known to cause limb malformations. The way in which they function and the type and severity of their effect differ per substance. To prevent teratogens from entering the market and causing limb malformations, it is important to perform predictive developmental toxicity tests. Currently, these tests move from the conventional costly and low-throughput *in vivo* animal models to more high-throughput models that reduce animal usage, and can be used to investigate the mechanisms by which teratogens alter normal limb development. In the future these methods have to deal with the increasing challenge to provide data on compounds to which humans may be exposed, but for which we lack developmental toxicity data.

Introduction

In a survey in the US, around 80% of pregnant women reported using a drug or nutritional supplement during pregnancy, with an average of four different types (Shum, Coleman, Hatakeyama, & Tuan, 2003). These drugs and nutritional supplements are potential teratogens that can adversely influence the normal development of the child. Besides chemical agents, infectious diseases, physical agents, recreational drugs, and chronic diseases could lead to adverse effects in the child (Shum et al., 2003). Adverse effects caused by teratogens are dependent on timing of exposure, because the susceptibility differs according to the different stages of embryonic development (Shum et al., 2003). The effects of teratogens are largest during organogenesis (Shum et al., 2003). Teratogens disrupt cellular processes by altering gene expression or interfering with signalling pathways. Individuals have different genotypes and therefore teratogens can lead to a range of effects and severity which differ among individuals, also influenced by the dose of the teratogen (Shum et al., 2003). Adverse effects that appear after exposure can cause major problems later in life or even lead to fetal death (Wilkie, 2003).

Limb malformations are the most visible and also the most frequently described phenotypic effect of teratogens (Holmes, 2002). The effects of teratogens on limb development range from abnormal, loss of, or additional elements of the limbs (Zuniga, Zeller, & Probst, 2012). Limb malformations are not life threatening, but they are often an indicator of more underlying adverse effects that were acquired during organogenesis (Holmes, 2002; Shum et al., 2003). The most commonly known teratogen that affects normal limb development is thalidomide, a substance used to relieve morning sickness (Zuniga et al., 2012). Among the more than 10 000 children that were affected by thalidomide 90% developed limb malformations (Vargesson, 2009). Current predictive developmental toxicology tests are largely influenced by this tragedy (Wilkie, 2003).

This article reviews the current state of knowledge on normal limb development. Furthermore, a number of teratogenic substances and their mechanistic effects on the developing limb are described. Lastly, the future of predictive toxicity testing in the limb, that moves from low-throughput, costly, *in vivo* animal research to more high-throughput, *in vitro* models, is discussed.

Limb development

1. Limb bud development

Around 27 days after fertilization the limbs start to develop in humans (Fig. 1) (Al-Qattan, Yang, & Kozin, 2009; Dimitrov, 2010). Along the body's antero-posterior axis *HOX* gene expression regionalizes the lateral plate mesoderm (Wolpert et al., 2007). WNT proteins are produced and secreted by the mesoderm and they regulate the expression and maintenance of the fibroblast growth factor (FGF) (Wolpert et al., 2007). FGF10 is expressed in the intermediate mesoderm at the site of limb development and is essential for the initiation of the limb bud (Shum et al., 2003). *HOX* genes determine where limbs develop by specifying where WNTs, and thus also FGFs, are expressed. Also the T-box transcription factors TBX4 and TBX5 are important in limb bud initiation via FGF (Wolpert et al., 2007). The limb bud consists of undifferentiated mesenchymal cells from the lateral plate covered in ectoderm (Al-Qattan et al., 2009; Dimitrov, 2010; Shum et al., 2003). On the tip of the limb bud, on the edge of ventral and dorsal ectoderm, lies the signalling centre called the apical ectodermal ridge (AER) (Al-Qattan et al., 2009; Wolpert et al., 2007). Another signalling centre is the zone of polarizing activity (ZPA), which is positioned on the posterior side of the limb bud (Wolpert et al., 2007). These different signalling centres control the outgrowth and patterning of the limb in three dimensions (Schwabe & Mundlos, 2004). The establishment and maintenance of these centres is controlled by FGF (Wolpert et al., 2007).

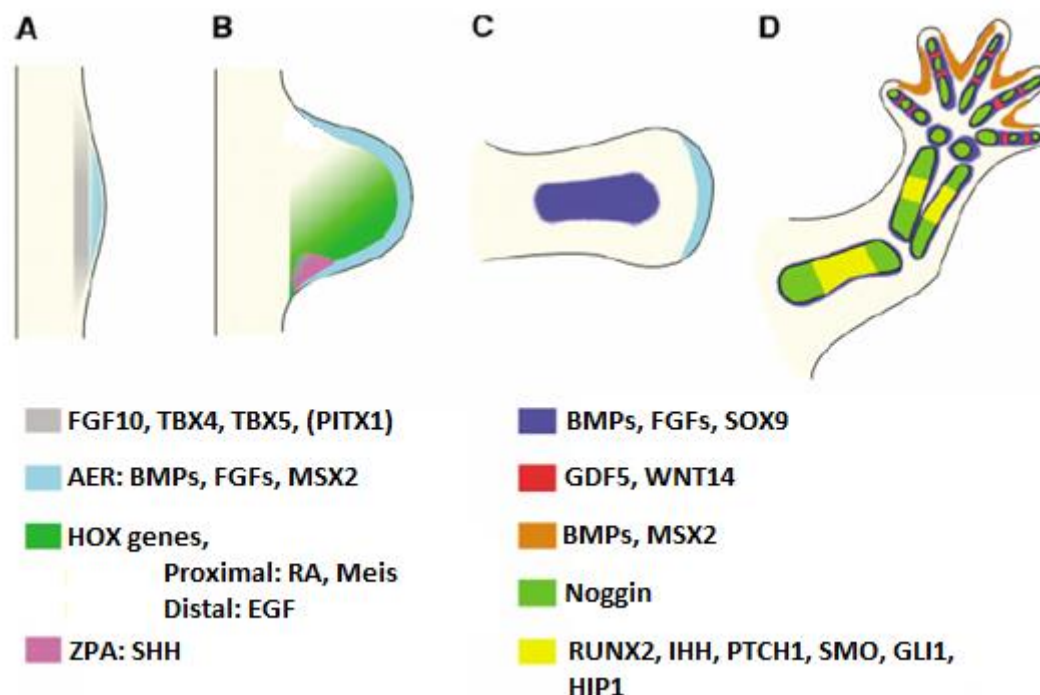


Figure 1. Embryonic limb development. **A** Limb bud initiation. Signals from the underlying mesenchyme initiate development of the limb and AER in the ectoderm. **B** The limb bud starts to grow, and patterning of the axis is established, under the influence of signals from the AER, ZPA, and underlying mesenchyme. **C** Cells condensate to start bone formation, and further patterning of the axis. **D** Differentiation into bone, joints, and cartilage. Meanwhile, digits are formed by apoptosis in the interdigital regions (adapted from Shum et al., 2003).

2. Limb Patterning

The upper and lower limbs share most of their processes that establish the patterning of the limbs in three dimensions (Dimitrov, 2010). The proximo-distal axis runs from the base of the limb to the tip of the hand (Fig. 2). The antero-posterior axis runs parallel with the body axis from thumb to little finger. Finally the dorso-ventral axis, runs from the back of the hand to the palm (Wolpert et al., 2007). Besides the similarities between the processes in the hind- and fore limb there are also some differences. Both *TBX5* and *TBX4* are expressed, but *TBX4* expression is downregulated in the forelimbs. While *PITX1*, a gene expressed in the hind limbs, upregulates *TBX4* expression there (Dimitrov, 2010).

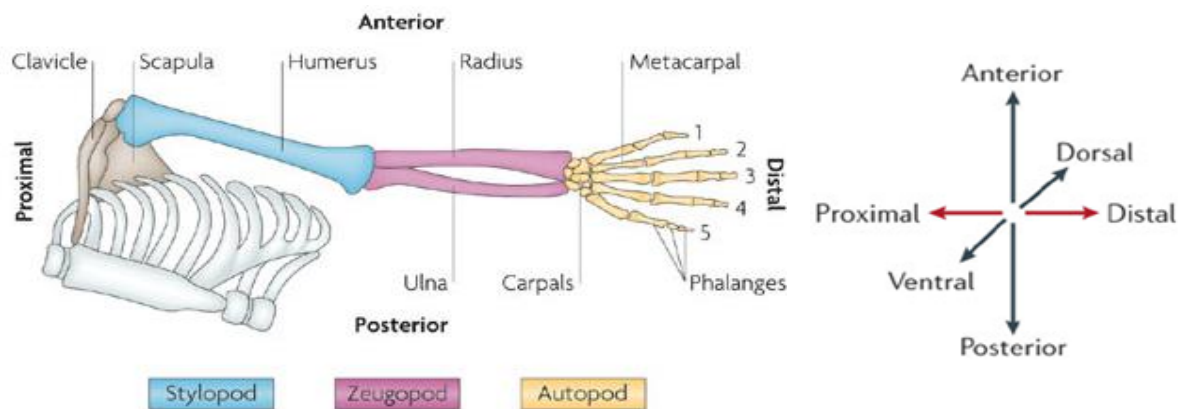


Figure 2. The three developmental axes of the limb are proximo-distal, antero-posterior, and dorso-ventral. (adapted from Tickle, 2005; Zeller, López-Ríos, & Zuniga, 2009)

Proximo-distal axis development

The AER is formed by the expression of *BMP4* in the mesenchyme (Zuniga et al., 2012). The AER is essential for limb outgrowth and patterning, by expressing *FGFs*, *BMPs* and *MSX2* (Al-Qattan et al., 2009; Dimitrov, 2010; Schwabe & Mundlos, 2004; Shum et al., 2003; Wolpert et al., 2007). *FGF10* expression in the early limb bud maintains the AER by inducing the expression of *FGFs* (Al-Qattan et al., 2009). *FGF8*, *FGF4*, *FGF2*, *FGF9*, *FGF17*, expressed in the AER, maintain the proliferating and undifferentiated state of the mesenchymal cells in the underlying progress zone (Dimitrov, 2010). *FGF8* is expressed in the entire AER, and *FGF4* only in the posterior region (Wolpert et al., 2007). Cells leaving the progress zone differentiate, proceeding distally as the limb extends (Wolpert et al., 2007).

The most proximal structures seem to be patterned by a different process than the more distal structures. Retinoic acid (RA) is involved in patterning these more proximal structures by targeting genes of the *MEIS* family. This process is blocked distally by expression of epidermal growth factor (EGF) (Zuniga et al., 2012).

Antero-posterior axis development

FGF4 expressed in the AER is in a positive feedback loop with the signalling molecule Sonic hedgehog (SHH) of the ZPA (Fig. 3)(Al-Qattan et al., 2009; Shum et al., 2003; Wolpert et al., 2007). Expression of these signalling molecules maintains the presence of these two centres (Al-Qattan et al., 2009; Shum

et al., 2003; Wolpert et al., 2007). Expression of SHH provides the positional information along the antero-posterior axis in a dose-time dependent manner (Al-Qattan et al., 2009). SHH expression is activated by *dHAND* and *5'-HOX* in the posterior limb bud mesenchyme. SHH blocks the conversion of the long active GLI3 molecule to the repressor GLI3R, resulting in a low concentration of GLI3R close to the ZPA and increasing in anterior direction. Expression of GLI3R and ALX4 restricts the expression of *dHAND* and *5'-HOX* to the posterior side and thereby also SHH expression (Dimitrov, 2010). This results in an expression gradient of SHH, with high concentrations close to the ZPA. Combined with cholesterol promoted diffusion by SHH, this gives the cells positional information (Dimitrov, 2010). SHH induces differentiation and digit formation by activating BMPs, GLI1 and PTCH1 (Dimitrov, 2010). Cells can then interpret their position by differentiating into a particular structure or digit according to the concentration of the morphogen (Shum et al., 2003; Wolpert et al., 2007). For instance, SHH activates Gremlin (GREM1) that blocks BMP activity. BMP is needed to maintain the polarizing zone. The AER is also dependent on the progress zone for its maintenance and functioning. A high concentration of BMP would lead the AER to regress via cell death and a low concentration of BMP would result in a wider AER and more digits (Wolpert et al., 2007). *MSX2* is expressed in the interdigital mesenchymal cells and regulates apoptosis to form individual digits (Shum et al., 2003). *HOX* genes are also probably involved in giving positional information along the limb axis. The expression of the *HOX* genes is variable as the limb bud grows (Shum et al., 2003; Wolpert et al., 2007).

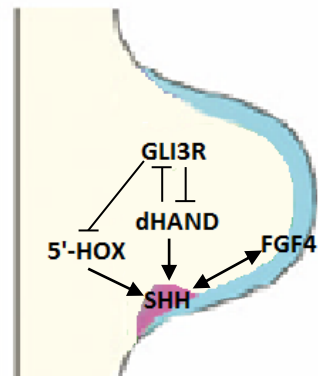


Figure 3. Activation of SHH signaling: the interactions controlling activation of *SHH* expression in the posterior mesenchyme are shown. (Adaptation from Zuniga et al., 2012)

Dorso-ventral axis development

The dorso-ventral axis runs from the back of the hand to the palm and specifies the muscle, tendons and nails to form according to their position (Schwabe & Mundlos, 2004; Wolpert et al., 2007). *WNT7a* is expressed in the dorsal ectoderm, and induces the expression of the LIM homeobox transcription factor (*LMX1*) in the underlying mesenchyme (Fig. 4) (Al-Qattan et al., 2009; Dimitrov, 2010; Shum et al., 2003; Wolpert et al., 2007). *WNT7a*, via *LMX1* patterns the dorsal identity. The transcription factor engrailed (*EN1*) limits expression of *WNT7a* to the dorsal ectoderm and therefore specifies ventral identity (Al-Qattan et al., 2009; Dimitrov, 2010; Schwabe & Mundlos, 2004; Wolpert et al., 2007). *BMP* controls *EN1* expression, and is also said to have an independent role in ventralization (Dimitrov, 2010).

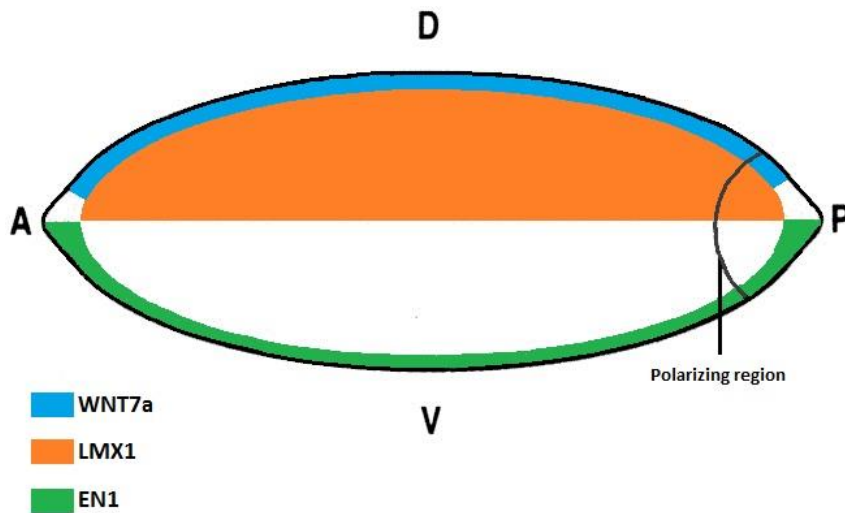


Figure 4. Transverse section through the limb bud, depicting the genes involved in patterning the dorso-ventral axis. (adapted from Wolpert et al., 2007)

3. Bone and cartilage formation

The positional information given by the patterning of the axis determines the place of bone and cartilage formation (Shum et al., 2003). Bone and cartilage formation is a two step process. First the cells of the mesenchyme condensate forming a preskeletal blastema (Al-Qattan et al., 2009). Condensation is regulated by BMPs and FGFs (Al-Qattan et al., 2009; Dimitrov, 2010). The second process is differentiation into either cartilage forming chondrocytes or boneforming osteoblasts. Differentiation of osteochondral cells is also influenced by both BMPs and FGFs (Dimitrov, 2010). Chondrification occurs in a proximal to distal direction under the influence of mainly *SOX9* (Al-Qattan et al., 2009). Chondrocytes are recognizable by the expression of *Noggin* (Shum et al., 2003). Repression of chondrification by *GDF5* and *WNT14* leads to joint formation (Al-Qattan et al., 2009; Wolpert et al., 2007). The process of chondrification occurs earlier in development than the process of ossification (Dimitrov, 2010). Ossification is mainly regulated by expression of *RUNX2*. *RUNX2* is regulated by *IHH* through mediation of the signalling molecules *PTCH1* and *SMO*, and the genes *GLI1* and *HIP1* (Dimitrov, 2010).

4. Muscle formation

The cells that will form the muscle migrate from the somites, into the limb bud. Expression of *TCF4* in the limb mesenchyme attracts the migrating myoblasts (Wolpert et al., 2007). They either enter the ventral or dorsal extending limb, and cannot cross the midline, defined by expression of *LMX1b*. In this phase the myoblasts express *HOXA11*. Next, the cells divide to form a dorsal and ventral block of presumptive muscle and start expressing *HOXA13* (Wolpert et al., 2007). *PAX3* expression in this phase prevents the cells from differentiating. The overlying ectoderm controls expression of *PAX3* and therefore premature differentiation, by expressing *BMP4* (Wolpert et al., 2007). Superficial

muscles develop before the deeper muscles and development moves from proximal to distal (Al-Qattan et al., 2009). The development of tendons is marked by expression of the transcription factor Scleraxis (Wolpert et al., 2007).

5. Vascular development

Angioblasts derived from somites migrate to the limb buds to form blood vessel networks by angiogenesis. Angiogenesis is the remodelling of pre-existing vessels to new capillaries, for vessel stabilization or maturation (Knudsen & Kleinstreuer, 2011). As the limb bud grows, the hypoxic cells in the limb bud signal to the endothelial cells at the tip of blood vessels to start angiogenesis, by expressing VEGF. The binding of VEGF and also FGF2 to the endothelial cells activates a G-protein signalling cascade and other proteases to degrade the vessel wall to allow sprouting along the VEGF gradient using filopodia (Cross & Claesson-Welsh, 2001). Tip cells express DLL4 that can bind to Notch on adjacent endothelial cells. This leads to downregulation of the VEGF-receptor, which stimulates proliferation. These proliferating cells produce FLT1 that binds VEGF to prevent overgrowth and vascular deregulation. The vascular fusion of adjacent vessels in this developing network, is facilitated by macrophages that are attracted to the site by expression of CCL2 in the tip cells. Mural cells are attracted to the vessels to bring structural support. Interaction between the endothelial cells and the mural cells leads to stabilization of the vascular network and activates TGF β , which inhibits proliferation and stimulates differentiation of the mural cells (Knudsen & Kleinstreuer, 2011).

Teratogens of limb development

Development of the limbs in humans starts around day 27 (week 4) until completion around day 55 (week 8) (Vargesson, 2009). During this time, development can be influenced by teratogens causing limb malformations and functional defects (Fig. 5). Exposure of the limbs earlier in the development has a larger effect than exposure later in the development (Shum et al., 2003). Also, malformations that are caused by exposure later in development of the limb, often occur more distally than after exposure earlier in development (Wolpert et al., 2007). This chapter discusses different teratogens and the mechanisms by which they influence limb development.

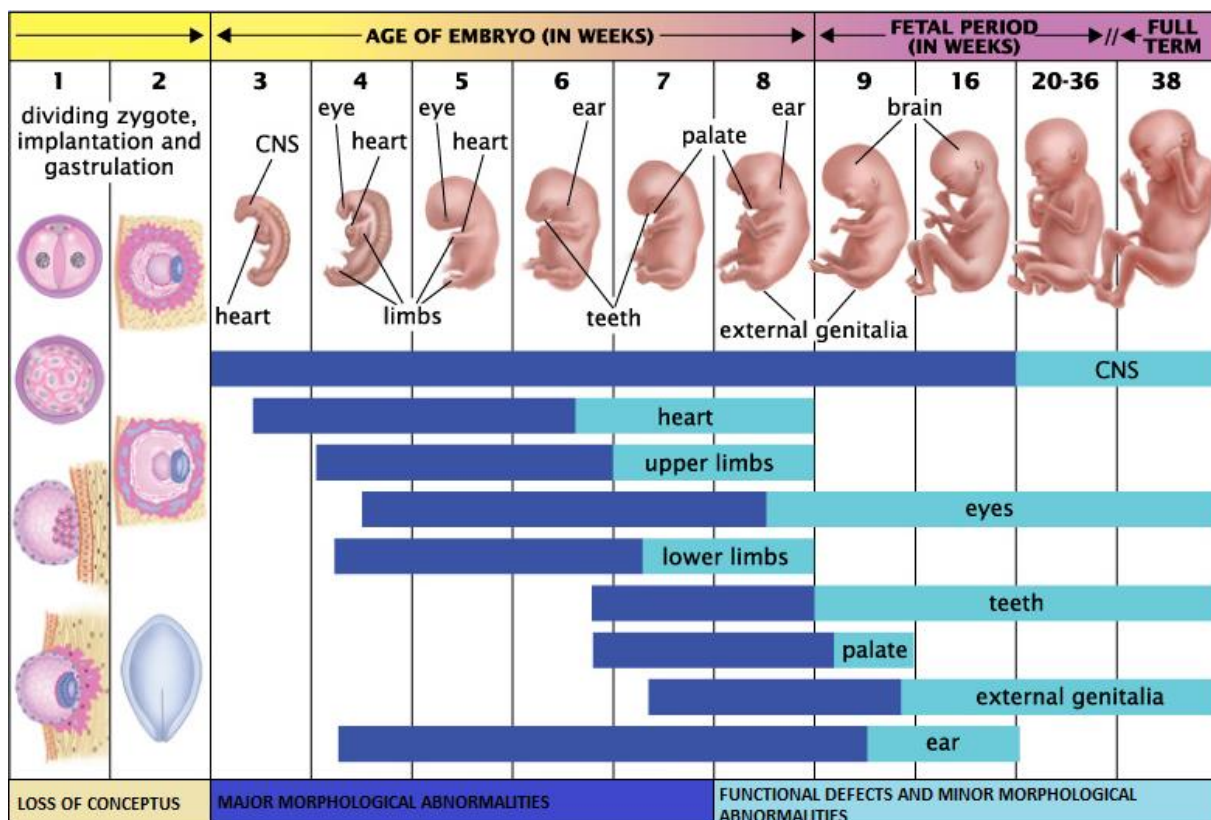


Figure 5. Fetal development over time, demonstrating the effects of teratogens on organogenesis. (adapted from from <http://www.slideshare.net/SDRTL/fetal-development-10766134>)

1. Thalidomide

Still today, the most commonly known teratogen is thalidomide (Shum et al., 2003). It was marketed in 1957 as a sedative, but was also prescribed to relieve morning sickness in the early pregnancy (Dela Cruz, Terry, & Matushansky, 2012; Knobloch, Shaughnessy, & Ruther, 2007; Vargesson, 2009; Zeller, 2010). The drug was banned in 1962, because of the developmental defects it caused. By this time already more than 10 000 children were born with severe birth defects (Dela Cruz et al., 2012; Vargesson, 2009). Around 90% of these children had limb malformations, like limb reduction (Vargesson, 2009). One of the reasons this tragedy could have happened is because rats and mice, used to test the drug before marketing, seemed completely unresponsive to thalidomide (Knobloch et al., 2007; Vargesson, 2009). Finding the mechanism by which thalidomide influences development

has proven to be difficult (Dela Cruz et al., 2012; Holmes, 2002; Knobloch et al., 2007; Knudsen & Kleinstreuer, 2011; Shum et al., 2003; Wilkie, 2003; Zeller, 2010). Besides answering the question why some species seem more affected than others, the mechanism must also explain how exposure in different time-windows causes different effects and explain the range of effects observed (Vargesson, 2009). The mechanism that seems to be answering some of these questions is the inhibition of angiogenesis (Knudsen & Kleinstreuer, 2011; Therapontos, Erskine, Gardner, Figg, & Vargesson, 2009; Vargesson, 2009). Thalidomide destroys newly formed angiogenic vessels and prevents the filipodial extensions necessary for sprouting (Therapontos et al., 2009; Vargesson, 2009). Cell death in the newly formed angiogenic vessels is acquired by a stress response in endothelial cells by activation of kinases (Knudsen & Kleinstreuer, 2011). A study by D'Amato et al (1994) performed a rabbit corneal micropocket assay to evaluate angiogenesis in the rabbit eye exposed to 200mg/kg thalidomide. They showed that angiogenesis mediated by FGF2 is reduced under the influence of thalidomide (D'Amato, Loughnan, Flynn, & Folkman, 1994). Thalidomide inhibits VEGF and FGF2 mediated sprouting by blocking the promoters of the proteases (Knudsen & Kleinstreuer, 2011). When thalidomide is taken during limb vascularisation and limb outgrowth, it results in severe limb malformations (Vargesson, 2009). If exposure to the drug is in a later stage of limb development it has minor effects on the limb development and effects occur more distally. During limb development other bloodvessels are already more mature, therefore less angiogenesis, and thalidomide doesn't influence them much. However, if the drug is taken during angiogenesis in earlier stages of development it leads to lethality of the embryo (Kleinstreuer et al., 2013; Vargesson, 2009). The latter is what led to believe that animals are unresponsive to thalidomide. There were no visible effects of thalidomide on rodents, because these animals were never born due to lethality of thalidomide (Vargesson, 2009).

An often seen limb defect in children exposed to thalidomide is phocomelia, where the proximal structures are absent, but the more distal structures are present (Fig. 6)(Vargesson, 2009). This can occur when after thalidomide exposure and cell death of the mesenchyme, still some signalling in the AER remains. This small amount of signalling can cause re-establishment of the AER, when exposure to thalidomide has disappeared, and thus formation of the more distal structures (Stephens, Bunde, & Fillmore, 2000; Therapontos et al., 2009; Vargesson, 2009).

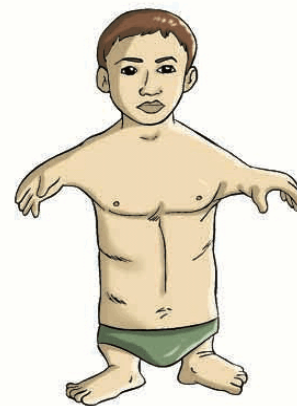


Figure 6. Drawing of an individual with phocomelia, a limb malformation often seen after exposure to thalidomide. (from: <http://wordinfo.info/words/images/phocomelus-man-color.gif>)

2. Retinoic acid

Retinoic acid (RA) is expressed in the normal developing limb. However, increased or decreased levels of RA can have dramatic effects on the development. In the first trimester of pregnancy RA exposure often results in limb reduction malformations in both humans (Die-Smulders et al., 1995; Lee, Kochhar, & Collins, 2004; Rizzo, Lammer, Parano, Pavone, & Argyle, 1991) and animals (Die-Smulders et al., 1995; Docterman & Smith, 2002; Lee, Liao, Shimizu, & Collins, 2010; Rizzo et al., 1991; Tsuiki & Kishi, 1999). This is a consequence of additional RA in regions where RA is expressed,

and RA in RA-sensitive regions that ought to be RA-free for critical windows of time (Docterman & Smith, 2002). A study by Qin et al. (2002) identified genes that potentially mediate the effects of RA, by microanalysis. They exposed pregnant mice to a high dose of 100 mg/kg RA at 11 days after fertilization and performed a microanalysis of the RNA expressed in the developing forelimb bud 3 – 24 hours later (Qin, Cimildoro, Kochhar, Soprano, & Soprano, 2002). Almost all mouse embryos developed phocomelia (Fig. 6). After RT-PCR they found that, as early as 3 hours after exposure, PBX and MEIS mRNA levels are elevated (Qin et al., 2002). RA functions to regulate expression of *MEIS* in the proximal regions of the developing limb bud (Docterman & Smith, 2002; Zuniga et al., 2012). MEIS transports PBX from the cytosol to the nucleus where they form a heterodimer and function as a transcription factor (Docterman & Smith, 2002). This indicates that in the case of increased RA, RA will be expressed distally, leading to expression of MEIS and transport of PBX to the nucleus. These signals override the signals of FGF and BMP to maintain the AER and progress zone, and thus further development of the distal limb is halted (Docterman & Smith, 2002).

3. Alcohol

The use of alcohol during pregnancy can lead to Fetal Alcohol Spectrum Disorders (FASD). Besides the commonly known consequences of FASD, like craniofacial malformations, it can also cause limb defects (Johnson, Zucker, Hunter, & Sulik, 2007). Most of the conditions are a result of mis-patterning of the AP-axis. The same limb defects are caused by an RA deficiency (Johnson et al., 2007). The study by Johnson et al. (2007) exposed pregnant mice at day 9 to ethanol and BMS-189453, a retinoic acid receptor antagonist, and evaluated the effects on limb morphology in the developing embryo at day 18. Both compounds led to postaxial ectrodactyly and cell death in the AER. Johnson et al. (2007) also performed a qPCR of the RNA expressed in the developing forelimb bud of the embryos. This showed decreases in expression of SHH and TBX5 after exposure to alcohol. SHH is necessary for maintaining the AER and ZPA, and RA is an upstream regulator of SHH (Johnson et al., 2007). They also exposed mice to a combination of ethanol and RA. Exposure to RA in alcohol exposed mice restored normal limb development (Johnson et al., 2007). This supports the belief that alcohol induced limb defects are RA mediated.

Metabolism of ethanol depends on the enzymes alcohol dehydrogenase and aldehyde dehydrogenase (ALDH). The same enzymes are needed for the synthesis of retinoic acid (Johnson et al., 2007; Shum et al., 2003). The mechanism by which alcohol functions to cause limb malformations is explained by the competition for ALDH. Competition for ALDH ultimately leads to reduced levels of RA. Another mechanism is the deregulation of RA-receptors (Johnson et al., 2007).

4. Warfarin

Warfarin is an anticoagulant, used since the 1950's for its potential to prevent blood clotting in thrombosis (Holmes, 2002; Menger, Lin, Toriello, Bernert, & Spranger, 1997; Sathienkijkanchai & Wasant, 2005; Van Driel et al., 2000). Taking warfarin during the first trimester of pregnancy can

have adverse effects on skeletal development (Van Driel et al., 2000). Warfarin embryopathy encompasses shortening of the digits and ossification of the cartilaginous epiphyses (Holmes, 2002). The sensitive window for these effects is between 6-9 weeks after gestation (Holmes, 2002; Običan & Scialli, 2011).

Warfarin has an influence on the balance between bone- and cartilage formation (Yagami et al., 1999). A study by Thijssen et al. (1988) showed that levels of vitamin K in blood were reduced in rats exposed to 6µg of warfarin (Thijssen, Baars, & Vervoort-Peters, 1988). Vitamin K reductase is inhibited by warfarin, reducing the amount of circulating vitamin K (Holmes, 2002; Menger et al., 1997; Običan & Scialli, 2011; Pauli, Lian, Mosher, & Suttie, 1987; Sathienkijanchai & Wasant, 2005). Vitamin K is a coenzyme for the synthesis of matrix GLA protein and osteocalcin (Holmes, 2002; Sathienkijanchai & Wasant, 2005; Yagami et al., 1999). Reducing circulating vitamin K leads to reduced synthesis of GLA protein and osteocalcin. These proteins are present in high levels in cartilage and bone (Yagami et al., 1999). GLA protein and osteocalcin function to inhibit mineralization of cartilage into bone (Yagami et al., 1999). Warfarin thus decreases the inhibition of mineralization by inhibiting vitamin K reductase and this leads to premature mineralization.

5. Valproic acid

Valproic acid is an anticoagulant used for the treatment of epilepsy and as a mood stabilizer (Faiella et al., 2000; Holmes, 2002; Kostrouchova, Kostrouch, & Kostrouchová, 2007; Kozma, 2001; Massa, Cabrera, Menegola, Giavini, & Finnell, 2005; Wiltse, 2005). It was first on the US market in 1978. Since then, exposure of valproic acid during pregnancy has been related to a 3.59 times increased occurrence of limb defects (Holmes, 2002; Kozma, 2001). When taken during the first trimester of pregnancy, valproic acid can cause defects in both fore- and hind limbs, for instance shortened digits, absence of distal radius, shortened fibulae, bowed tibia and abnormal knee joints (Faiella et al., 2000; Kozma, 2001). Valproic acid also causes other major developmental malformations besides limb defects, like spina bifida (Faiella et al., 2000; Holmes, 2002; Kostrouchova et al., 2007; Massa et al., 2005; Wiltse, 2005). Since valproic acid causes a large range of possible outcomes, the affected gene must be of major importance in limb development. The malformations indicate that after exposure to valproic acid the specification of the antero-posterior axis is affected (Kostrouchova et al., 2007). Research in animal models confirms this belief by indicating altered *HOX* gene expression as the mode of action (Faiella et al., 2000; Holmes, 2002; Kostrouchova et al., 2007; Kozma, 2001). The study by Faiella et al. (2000) showed that after exposure to 50 µg/ml valproic acid to human embryonal carcinoma cells *HOX* expression was altered. RNase protection assays using probes corresponding to human homeobox genes indicated that valproic acid upregulates expression of *HOXd1*, *HOXd8*, and *HOXd10*, and downregulates expression of *HOXd11* and *HOXd12* (Faiella et al., 2000). Since *HOX* gene expression is essential for limb development and it varies over time, consequences of valproic acid exposure could vary greatly over the course of development. Another postulated mechanism for the range of effects caused by valproic acid exposure is the altered expression of WNT signalling (Wiltse, 2005). Valproic acid increased levels of β-catenin and LEF-TCF in neuro2A mouse cells (Phiel et al., 2001). Both β-catenin and LEF-TCF are part of the WNT signalling pathway (Wiltse, 2005).

Application of alternative models in limb (mal)development research

The traditional method for predictive toxicity testing in developmental research is the use of *in vivo* animal models (Kavlock et al., 2012; Knudsen et al., 2011; Knudsen & DeWoskin, 2011; Knudsen & Kleinstreuer, 2011; Piersma, 2006). Pregnant dams of two species, usually rats and rabbits, are exposed to potential teratogens during organogenesis and the effects on mother and fetus are evaluated (Knudsen & Kleinstreuer, 2011; Knudsen & DeWoskin, 2011; Knudsen et al., 2011). The use of two species has been introduced to enhance the chances of detecting human teratogens. The advantage of this *in vivo* test situation is that it represents full development. Any alternative assay would only represent a part of development and maternal environment (Piersma, 2006). The interaction among different regulatory mechanisms and pattern formation are crucial in development and *in vivo* animal research represents the human situation in a way that has been proven effective in assessing developmental toxicity and in preventing false negatives (Kavlock et al., 2012). Although *in vivo* animal research has the advantage of representing a true developing environment there are also some downsides to this method. The effects of a substance are based on phenotypic outcomes (Kavlock et al., 2012; Knudsen & DeWoskin, 2011; Knudsen et al., 2011; Singh, Knudsen, & Knudsen, 2005). This gives no information on how the substance functions to cause this effect. For instance the limb malformation phocomelia, can be caused by both thalidomide and retinoic acid, by two different mechanisms. Another disadvantage is that it's hard to extrapolate outcomes of *in vivo* animal research to other species, doses, or life stages. Lastly, other disadvantages are the costs, time and throughput of the method. The costs are high in both animal- and financial resources (Knudsen et al., 2011). It takes time to do *in vivo* animal research, because you have to wait until the phenotype is expressed. Finally the throughput of animal research is low (Kleinstreuer et al., 2013; Knudsen & Kleinstreuer, 2011; Knudsen et al., 2011). This is a major challenge for the future, because *in vivo* animal research cannot compete with the high demand for toxicity data. The current EU initiative for Registration, Evaluation and Authorisation of Chemicals (REACH), aims to collect toxicity information on the >80 000 chemical compounds to which humans may be exposed and for which we lack developmental toxicity data (Knudsen & DeWoskin, 2011; Piersma, 2006). Therefore the development of higher-throughput models that use reduced amounts of animals or no animals, is urgent. The promising alternative methods are genomics technology, embryonic stem cells (ESC), zebrafish embryos (ZFE), and computational models. In this chapter these technologies are discussed, and also how they can be used to assess limb development toxicity is evaluated.

Genomics technology is used to identify critical genes for development and also to identify patterns of gene expression changes that are characteristic for classes of compounds (Knudsen et al., 2011; Piersma, 2006). The latter, is to form gene expression signatures that can be interpreted to predict the embryotoxic effects at the morphological level (Knudsen et al., 2011; Piersma, 2006). This makes this method faster than animal research, because in animal research phenotypic outcome is evaluated, and interference from a chemical with normal gene expression is likely an early effect on the route to malfunction. Therefore, changes are spotted earlier and probably also more relevant changes are spotted that do not necessarily result in phenotypic changes, but can have a large

impact. Also, this method has the ability to track temporal patterns of morphological changes with a concurrent change in responses of genes (Knudsen et al., 2011). An example of a study that uses genomics technology for the use of detecting the mechanism behind exposure to a teratogen on limb development is the previously mentioned study by Qin et al. (2002). To identify the genes that potentially mediate the effects of retinoic acid on limb development, they performed a microarray analysis of RNA isolated from mouse forelimbs exposed to retinoic acid on day 11 after fertilization (Qin et al., 2002). This type of study can also be used to assess the expression pattern of potential limb development teratogens.

For embryonic stem cells, the potential embryotoxicity of xenobiotics is assessed by its interference with the cell differentiation of permanent embryonic stem cell lines and comparing differences in sensitivity between embryonic and adult tissues to cytotoxic damage (ECVAM, april, 2014). This gives an indication of the embryotoxicity for pre-implantation stages of mammalian development. It also exploits the advantage of cell-cell interaction and conservation of cell signalling to detect dose-response activity of compounds on biological pathways and cellular processes that are critically important for morphogenesis and differentiation (Knudsen et al., 2011). A study by zur Nieden et al. (2003) shows that embryonic stem cell testing can be used to assess the potential chemical effects on osteogenic, and chondrogenic differentiation (zur Nieden, Kempka, & Ahr, 2004). As described earlier, the formation of bones and cartilage are important steps in limb development, and deregulation of this process can cause limb malformations, like in the case of warfarin. To assess a compounds potential to interfere with differentiation into bone or cartilage, changes in marker gene expression for the different tissues can be quantified by real-time PCR (Zur Nieden et al., 2004).

The usage of zebrafish embryos can assess the extent to which fine-scale phenotypic changes result from exposure to xenobiotics (Knudsen et al., 2011). Tracking the movements and history of individual cells in a growing embryo, by using sophisticated cell imaging technologies, provides the opportunity to assess early biological consequences of disruption due to teratogens (Knudsen et al., 2011; Knudsen & DeWoskin, 2011). Therefore this technique can give more detail about the underlying cellular mechanisms that appear after exposure than genome-based technologies can. Zebrafish embryos have been used in the search for the mechanisms on limb maldevelopment behind thalidomide exposure. A study by Therapontos et al. (2009) exposed zebrafish embryos to CPS49, a tetrafluorinated analogue of thalidomide, and used time-lapse imaging to analyse the development of intersomitic blood vessels over a 3hours period. They found that CPS49 decreased the number of tip cell filopodia and actin-rich structures that are necessary for endothelial cell migration and outgrowth (Therapontos et al., 2009). A study by Selderslaghs et al. (2009) showed that this technique can also be used as a screening assay to identify teratogenic and embryotoxic chemicals for limb development (Selderslaghs, Van Rompay, De Coen, & Witters, 2009).

The previously described models all have the challenge of extrapolating the information given by these tests to a complete developing mammal (Knudsen & DeWoskin, 2011). Computational models combine the information from all these different tests to create an overview of the biological system (Kongsbak, Hadrup, Audouze, & Vinggaard, 2014). A database that has combined all these types of data is the ToxCast™ database (Kavlock et al., 2012). The ToxCast™ database encompasses data on molecular and cellular pathways that are chemical targets. This gives information on how chemicals cause toxicity rather than just reporting potential phenotypic outcomes. The database combines information on a chemical's properties, dose, time of exposure, maternal and embryonic genetic

makeup and its effects on signalling interactions (Kavlock et al., 2012). This meta-analysis of different data creates a dataset that is a more powerful description of normal developmental processes and toxicity than individual data (Singh et al., 2005). Computational models can be used to identify potentially toxic chemicals, prioritize existing chemicals for *in vivo* testing, and evaluate pathogenesis of existing compounds (Kavlock et al., 2012; Knudsen & DeWoskin, 2011; Kongsbak et al., 2014). Predictive models attempt to simulate the biological response that results from exposure to a compound, by creating a virtual embryo (Knudsen & DeWoskin, 2011; Knudsen et al., 2011). An example of this is a study by Kleinstreuer et al. (2013) who used the ToxCast™ database to build a predictive model for vascular development. Vascular development is an important part of normal limb development and can be disrupted by thalidomide exposure. They've built a model consisting of a number of critical cell types and molecular signals needed for vasculogenesis and angiogenesis (Kleinstreuer et al., 2013). Building a model to predict the toxicity of potential vascular development disruptors is important because of the diversity of phenotypes that can arise from vascular disruption (Kavlock et al., 2012). Evaluating only *in vivo* animal research data does not necessarily indicate vascular disruption, like in the case of thalidomide, where discovering the underlying mechanism took more than 50 years. The ToxCast™ database was searched to identify targets of vasculogenesis and/or angiogenesis by evaluating over 600 high-throughput assays (Knudsen & Kleinstreuer, 2011). The potential molecular targets were further expanded with literature on vasculogenesis and angiogenesis to build the model (Kleinstreuer et al., 2013). In this particular model the focus is the "autonomous" behaviour of cells and its reactions to chemicals that can lead to changes in vasculogenesis and/or angiogenesis. Cells in this model represent an approximate distribution of cell types involved in vasculogenesis and angiogenesis in both the mammalian embryo and the placenta. The model contains endothelial tip cells, endothelial stalk cells, inflammatory cells, mural cells, apoptotic cells and the extracellular matrix (Kleinstreuer et al., 2013). The extracellular matrix remains static during the simulation. Furthermore, there were angiogenic signals that were represented as biochemical fields and angiogenic signals that were represented by their influence on cellular behaviours, such as adhesivity, motility and proliferation. Each cell also receives information on the neighbouring cells, and this can influence cellular behaviour. At the start of the simulation the spatial distribution of different cell types differed per run, however the relative distribution of the different cell types remained constant for every simulation (Kleinstreuer et al., 2013). The model was run first without exposure to a compound to represent normal vascular development (Fig. 7a). The simulation showed embryonic vascular plexus formation and then remained at a steady state (Kleinstreuer et al., 2013). To evaluate this model for predictive toxicity purposes the compound 5HPP-33 was added to the model (Fig. 7b-d). 5HPP-33 is a thalidomide derivative and is a good reference compound because of the high amount of information on this compound in both *in vitro* and *in vivo* studies. Running the model with the added 5HPP-33 compound showed that 5HPP-33 affected the angiogenic switch via downregulation of the VEGF receptor and the chemokine pathway via downregulation of inflammatory signalling molecules (Kleinstreuer et al., 2013). It also targeted vessel remodelling via downregulation of TGFβ and extra cellular matrix interactions. The results of this study also hints towards an effect of 5HPP-33 on VEGF transcription and growth factor signalling via an endocrine-regulated pathway. The concordance between the data from previous research and the output of the computational model indicate that computational models can be a good predictor of toxicology (Kleinstreuer et al., 2013). The predictive toxicity research using computational models is still in its infancy, but when in due time a complete virtual embryo is created, this method will save a lot of time, money and animal lives (Knudsen & DeWoskin, 2011; Knudsen et al., 2011).

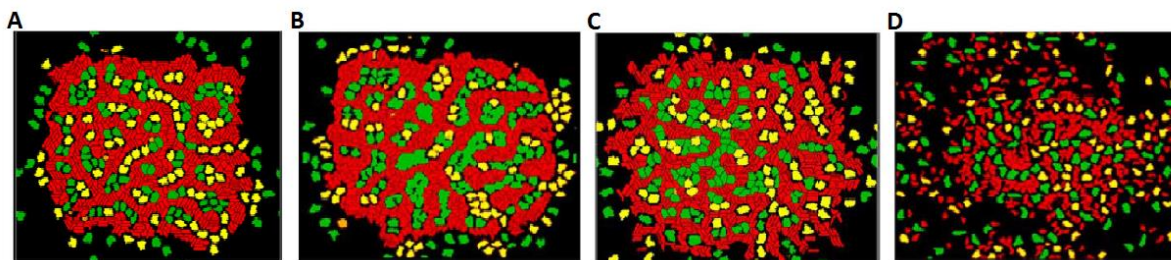


Figure 7. Normal vascular development visualized with a computational vascular development model (A), and a range of possible effects of 5HPP-33 (B-D) on the outcome of the model at 3h. (adapted from Kleinstreuer et al., 2013)

Currently, six alternative assays have been validated and are being used to replace, reduce and refine animal research (ECVAM, april, 2014). One of these six is the embryonic stem cell test. Then there is also the *in vitro* Syrian hamster embryo cell transformation assay (SHE CTA), for testing the carcinogenic potential of chemicals. The embryotoxicity test in post-implantation whole embryo cultures (WEC), evaluates the potential embryotoxicity by interference with differentiation and development of whole embryos in *in vitro* cultures. There is also the lung cell assay, that assesses potential embryotoxicity by monitoring the effect of the test compound on total protein synthesis and DNA synthesis in cultured human fetal lung fibroblasts. The rabbit articular chondrocyte functional toxicity test, determines the toxicity by the compounds effect on the production of proteoglycan by rabbit articular chondrocytes. Lastly, also a limb specific test is validated. The limb bud micromass test assesses potential teratogenicity of chemicals by its interference with growth, migration and re-aggregation of dissociated primary cells of rat limb buds (ECVAM, april, 2014).

These methods can be used to investigate the mechanisms of limb development further and evaluate the influence of potential teratogens on limb development. Although these methods are alternatives to animal research, they currently cannot replace animal research, but can be used as pre-screening prior to *in vivo* animal research (Piersma, 2006). Hereby, they may reduce the number of animal research tests that have to be performed, leading to increased efficiency.

Discussion

Normal development can be deregulated by exposure to teratogens. An important factor herein is the timing of exposure. Exposure earlier in development leads to embryonic loss or major morphological abnormalities (Shum et al., 2003). Functional defects and minor morphological abnormalities may be the consequence of exposure to teratogens later in development (Shum et al., 2003). A critical period for exposure to teratogens is during organogenesis (Shum et al., 2003). Limbs are an example of an organ that develops during organogenesis.

Normal limb bud development starts around 27 days after fertilization with cell proliferation of mesenchymal cells (Al-Qattan et al., 2009; Dimitrov, 2010). *HOX* genes determine where limbs develop by specifying where WNTs, and thus also FGFs, are expressed (Wolpert et al., 2007). Important in normal limb development and axis patterning are the AER and ZPA signalling centres (Al-Qattan et al., 2009; Wolpert et al., 2007). The AER is involved in proximo-distal axis patterning, by expressing mainly FGFs (Al-Qattan et al., 2009; Dimitrov, 2010). The ZPA is involved in patterning the antero-posterior axis, by expression of SHH (Al-Qattan et al., 2009). The dorso-ventral axis is defined by expression of WNT7a dorsally, and engrailed expression ventrally (Al-Qattan et al., 2009; Dimitrov, 2010; Schwabe & Mundlos, 2004; Shum et al., 2003; Wolpert et al., 2007). Bone and cartilage are formed from condensed mesenchymal cells under the influence of BMP and FGF (Dimitrov, 2010). Then cells either form cartilage, under expression of SOX9, or bone, under the expression of RUNX2 (Al-Qattan et al., 2009; Dimitrov, 2010). Expression of GDF5 and WNT14 lead to joint formation (Al-Qattan et al., 2009; Wolpert et al., 2007). Muscles are formed from migrating cells from the somites that are attracted by expression of TCF4 in the developing limb (Wolpert et al., 2007). They either enter ventrally or dorsally and start dividing (Wolpert et al., 2007). Dividing pre-muscular cells express HOXA13. Differentiation in this phase is temporarily inhibited by expression of BMP (Wolpert et al., 2007). Muscle formation moves in a proximal to distal direction and tendons are formed by expression of Scleraxis (Al-Qattan et al., 2009; Wolpert et al., 2007). Vascular development in the limb is formed from angiogenesis (Knudsen & Kleinstreuer, 2011). VEGF expressed in hypoxic cells induces vessel wall degradation of nearby vessels to allow sprouting into the limb (Cross & Claesson-Welsh, 2001). Vascular fusion of the developing vessels is obtained by expression of CCL2 to attract macrophages. Mural cells, important for vessel stability, are formed under the expression of TGF β (Knudsen & Kleinstreuer, 2011).

Although we have a general idea of how limb development progresses, it is important to know more about the mechanisms driving limb development. If we know how genes regulate normal development we can use this information to discover how teratogens deregulate development. Commonly known teratogens that influence normal limb development, from which the mechanism is examined, are thalidomide, retinoic acid, alcohol, warfarin and valproic acid. Thalidomide, used as a sedative and to relieve morning sickness, inhibits angiogenesis (Dela Cruz et al., 2012; Knobloch et al., 2007; Knudsen & Kleinstreuer, 2011; Therapontos et al., 2009; Vargesson, 2009). It does this by destroying newly formed angiogenic vessels (Therapontos et al., 2009; Vargesson, 2009). Cell death herein is accomplished by evoking a stress response by activation of kinases (Knudsen & Kleinstreuer, 2011). Also, thalidomide prevents filipodial extensions necessary for sprouting, by inhibiting VEGF and FGF2 mediated sprouting by blocking promoters of the proteases (Knudsen & Kleinstreuer, 2011;

Therapontos et al., 2009; Vargesson, 2009). Retinoic acid, necessary for normal development, can have adverse effects if it is increased in areas where RA expression is present, or if RA is present in areas that ought to be RA-free for critical windows of time (Docterman & Smith, 2002). This RA expression will lead to expression of MEIS and transport of PBX to the nucleus (Docterman & Smith, 2002; Qin et al., 2002). These signals override the signals of FGF and BMP to maintain the AER and progress zone, and thus further development of the distal limb is halted (Docterman & Smith, 2002). Alcohol can cause mispatterning of the antero-posterior axis similar to RA deficiencies (Johnson et al., 2007). This is because the enzymes needed for alcohol metabolism are also needed for synthesis of RA. Competition for ALDH ultimately leads to reduced levels of RA (Johnson et al., 2007; Shum et al., 2003). Another postulated mechanism is the deregulation of RA-receptors (Johnson et al., 2007). Warfarin used as an anti-coagulant can cause problems in skeletal development by disrupting the balance between bone- and cartilage formation (Holmes, 2002; Menger et al., 1997; Sathienkijkanchai & Wasant, 2005; Van Driel et al., 2000; Yagami et al., 1999). Warfarin inhibits vitamin K reductase, and thereby circulating vitamin K is reduced (Holmes, 2002; Menger et al., 1997; Običan & Scialli, 2011; Pauli et al., 1987; Sathienkijkanchai & Wasant, 2005). Vitamin K is necessary for matrix GLA protein and osteocalcin (Holmes, 2002; Sathienkijkanchai & Wasant, 2005; Yagami et al., 1999). Reduced matrix GLA protein and osteocalcin by exposure to warfarin lead to decreased inhibition of mineralization (Yagami et al., 1999). Valproic acid used as an anticoagulant for the treatment of epilepsy and as a mood-stabilizer affects specification of the antero-posterior axis by altering *HOX* gene expression (Faiella et al., 2000; Holmes, 2002; Kostrouchova et al., 2007; Kozma, 2001; Massa et al., 2005; Wiltse, 2005). Another postulated mechanism is altered WNT expression (Phiel et al., 2001; Wiltse, 2005).

Currently used technology to identify potential teratogens for limb development is *in vivo* animal research (Kavlock et al., 2012; Knudsen et al., 2011; Knudsen & DeWoskin, 2011; Knudsen & Kleinstreuer, 2011; Piersma, 2006). This has the advantage of evaluating the changes occurring after exposure in a full embryonic system, but the disadvantages weigh heavily on the advantages. Toxicity is based on phenotypic endpoints rather than mechanistic, it is costly in both animal and financial resources, time consuming and low-throughput (Kavlock et al., 2012; Knudsen & DeWoskin, 2011; Knudsen et al., 2011; Singh et al., 2005). This last disadvantage is proven a major challenge, because of the increasing request for developmental toxicity data on compounds we may already be exposed to (Knudsen & DeWoskin, 2011; Piersma, 2006). The development of higher-throughput alternatives for *in vivo* animal research is therefore important and also looks promising. However, these studies can at the time not replace *in vivo* animal research completely, however, they can be used as pre-screening tools to evaluate embryonic limb development toxicity (Piersma, 2006). This would lead to reduced numbers of animals used and increased efficiency. Bone and cartilage formation can be assessed by both the embryonic stem cell test and the limb bud micromass test (Spielmann et al., 2006). The advantage of the embryonic stem cell test is that it uses a permanent cell line and therefore no animals need to be sacrificed for predictive toxicity testing, also it exploits the advantage of cell-cell interaction, and is a high-throughput assay (Knudsen et al., 2011; Spielmann et al., 2006). The limb bud micromass test does make use of pregnant animals for testing and is favorable over embryonic stem cells if assessing specifically differentiation of limb bud cells into cartilage producing chondrocytes and the effects of a compound on this process (Spielmann et al., 2006). Vascular development and disruption of vascular development can be assessed by both the zebrafish embryo assay or computational models. The advantage of zebrafish embryo testing is the

amenability to genetic methods and cell imaging (Knudsen & DeWoskin, 2011). Together, this offers a means to image cellular dynamics during morphogenesis. A major drawback of zebrafish embryos is that they develop a fin rather than a limb, and this makes it hard to extrapolate findings to mammalian species (Knudsen & DeWoskin, 2011). Computational models do not have this drawback, because they thrive to resemble a full human embryo. The United States environmental protection agency (EPA) has started the Virtual Embryo Project (v-Embryo™), aimed to develop a model that uses high-tech computer modeling and collections of data, from the ToxCast™, ACToR, and Virtual Liver databases, combined with biological knowledge (EPA, United states Environmental Protection Agency, september, 2013). The v-Embryo™ project focuses initially on early eye-, vascular-, and limb development, because these embryo systems are well documented, susceptible to environmental factors, and simple enough to be simulated by computer models (EPA, United states Environmental Protection Agency, september, 2013). Another advantage of computational models is that they include data on pharmacokinetics and a chemical's properties (Kavlock et al., 2012). Computational models are however still in their infancy, and therefore it will take time before they will fully represent a human embryo, and it can be used to replace animal testing strategies (Knudsen & DeWoskin, 2011; Knudsen et al., 2011).

Hopefully, investing in high-throughput mechanisms to evaluate developmental toxicity will eventually lead to methods to deal with the high demand on toxicity data. Hopes for the future are that chemicals to which humans may be exposed and for which we lack developmental toxicity data will have all been tested, and that there are low cost, fast ways to do predictive developmental toxicity testing, so that women are able to safely use drugs and nutritional supplements during pregnancy.

References

- Al-Qattan, M. M., Yang, Y., & Kozin, S. H. (2009). Embryology of the upper limb. *The Journal of Hand Surgery*, 34(7), 1340-1350.
- Cross, M. J., & Claesson-Welsh, L. (2001). FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends in Pharmacological Sciences*, 22(4), 201-207.
- D'Amato, R. J., Loughnan, M. S., Flynn, E., & Folkman, J. (1994). Thalidomide is an inhibitor of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 91(9), 4082-4085.
- Dela Cruz, F., Terry, M., & Matushansky, I. (2012). A transgenic, mesodermal specific, Dkk1 mouse model recapitulates a spectrum of human congenital limb reduction defects. *Differentiation*, 83(4), 220-230.
- Die-Smulders, D., Christine, E., Sturkenboom, M. C., Veraart, J., Van Katwijk, C., Sastrowijoto, P., et al. (1995). Severe limb defects and craniofacial anomalies in a fetus conceived during acitretin therapy. *Teratology*, 52(4), 215-219.
- Dimitrov, B. (2010). Genetic studies in developmental skeletal and limb defects.
- Docterman, K. E., & Smith, S. M. (2002). Of meis and men: lessons from a microarray study of teratogen action. *Teratology*, 66(5), 217-223.
- ECVAM. (april, 2014). *EURL ECVAM database service on alternative methods to animal experimentation (DB-ALM)*.
- EPA, United states Environmental Protection Agency. (september, 2013). *The Virtual Embryo Project*.

- Faiella, A., Wernig, M., Consalez, G. G., Hostick, U., Hofmann, C., Hustert, E., et al. (2000). A mouse model for valproate teratogenicity: parental effects, homeotic transformations, and altered HOX expression. *Human Molecular Genetics*, *9*(2), 227-236. doi:ddd022 [pii]
- Holmes, L. B. (2002). Teratogen-induced limb defects. *American Journal of Medical Genetics*, *112*(3), 297-303.
- Johnson, C. S., Zucker, R. M., Hunter, E. S., & Sulik, K. K. (2007). Perturbation of retinoic acid (RA)-mediated limb development suggests a role for diminished RA signaling in the teratogenesis of ethanol. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *79*(9), 631-641.
- Kavlock, R., Chandler, K., Houck, K., Hunter, S., Judson, R., Kleinstreuer, N., et al. (2012). Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. *Chemical Research in Toxicology*, *25*(7), 1287-1302.
- Kleinstreuer, N., Dix, D., Rountree, M., Baker, N., Sipes, N., Reif, D., et al. (2013). A computational model predicting disruption of blood vessel development. *PLoS Computational Biology*, *9*(4), e1002996.
- Knobloch, J., Shaughnessy, J. D., Jr, & Ruther, U. (2007). Thalidomide induces limb deformities by perturbing the Bmp/Dkk1/Wnt signaling pathway. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *21*(7), 1410-1421. doi:10.1096/fj.06-7603com
- Knudsen, T. B., & DeWoskin, R. S. (2011). Systems modeling in developmental toxicity. *General, Applied and Systems Toxicology*,

- Knudsen, T. B., Kavlock, R. J., Daston, G. P., Stedman, D., Hixon, M., & Kim, J. H. (2011). Developmental toxicity testing for safety assessment: new approaches and technologies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *92*(5), 413-420.
- Knudsen, T. B., & Kleinstreuer, N. C. (2011). Disruption of embryonic vascular development in predictive toxicology. *Birth Defects Research Part C: Embryo Today: Reviews*, *93*(4), 312-323.
- Kongsbak, K., Hadrup, N., Audouze, K., & Vinggaard, A. M. (2014). Applicability of Computational Systems Biology in Toxicology. *Basic & Clinical Pharmacology & Toxicology*,
- Kostrouchova, M., Kostrouch, Z., & Kostrouchová, M. (2007). Valproic acid, a molecular lead to multiple regulatory pathways. *FOLIA BIOLOGICA-PRAHA-*, *53*(2), 37.
- Kozma, C. (2001). Valproic acid embryopathy: report of two siblings with further expansion of the phenotypic abnormalities and a review of the literature. *American Journal of Medical Genetics*, *98*(2), 168-175.
- Lee, G. S., Kochhar, D. M., & Collins, M. D. (2004). Retinoid-induced limb malformations. *Current Pharmaceutical Design*, *10*(22), 2657-2699.
- Lee, G. S., Liao, X., Shimizu, H., & Collins, M. D. (2010). Genetic and pathologic aspects of retinoic acid-induced limb malformations in the mouse. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *88*(10), 863-882.
- Massa, V., Cabrera, R. M., Menegola, E., Giavini, E., & Finnell, R. H. (2005). Valproic acid-induced skeletal malformations: associated gene expression cascades. *Pharmacogenetics and Genomics*, *15*(11), 787-800. doi:01213011-200511000-00004 [pii]

- Menger, H., Lin, A. E., Toriello, H. V., Bernert, G., & Spranger, J. W. (1997). Vitamin K deficiency embryopathy: a phenocopy of the warfarin embryopathy due to a disorder of embryonic vitamin K metabolism. *American Journal of Medical Genetics*, 72(2), 129-134.
- Običan, S., & Scialli, A. R. (2011). Teratogenic exposures. Paper presented at the *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 157. (3) pp. 150-169.
- Pauli, R. M., Lian, J. B., Mosher, D. F., & Suttie, J. W. (1987). Association of congenital deficiency of multiple vitamin K-dependent coagulation factors and the phenotype of the warfarin embryopathy: clues to the mechanism of teratogenicity of coumarin derivatives. *American Journal of Human Genetics*, 41(4), 566-583.
- Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., & Klein, P. S. (2001). Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *The Journal of Biological Chemistry*, 276(39), 36734-36741.
doi:10.1074/jbc.M101287200 [doi]
- Piersma, A. H. (2006). Alternative methods for developmental toxicity testing. *Basic & Clinical Pharmacology & Toxicology*, 98(5), 427-431.
- Qin, P., Cimildoro, R., Kochhar, D. M., Soprano, K. J., & Soprano, D. R. (2002). PBX, MEIS, and IGF-I are potential mediators of retinoic acid-induced proximodistal limb reduction defects. *Teratology*, 66(5), 224-234.
- Rizzo, R., Lammer, E. J., Parano, E., Pavone, L., & Argyle, J. C. (1991). Limb reduction defects in humans associated with prenatal isotretinoin exposure. *Teratology*, 44(6), 599-604.
- Sathienkijkanchai, A., & Wasant, P. (2005). Fetal warfarin syndrome. *J Med Assoc Thai*, 88(suppl 8), S246-50.

- Schwabe, G., & Mundlos, S. (2004). Genetics of congenital hand anomalies. *Handchirurgie Mikrochirurgie Plastische Chirurgie*, 36(2/3), 85-97.
- Selderslaghs, I. W., Van Rompay, A. R., De Coen, W., & Witters, H. E. (2009). Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. *Reproductive Toxicology*, 28(3), 308-320.
- Shum, L., Coleman, C. M., Hatakeyama, Y., & Tuan, R. S. (2003). Morphogenesis and dysmorphogenesis of the appendicular skeleton. *Birth Defects Research Part C: Embryo Today: Reviews*, 69(2), 102-122.
- Singh, A. V., Knudsen, K. B., & Knudsen, T. B. (2005). Computational systems analysis of developmental toxicity: design, development and implementation of a Birth Defects Systems Manager (BDSM). *Reproductive Toxicology*, 19(3), 421-439.
- Spielmann, H., Seiler, A., Bremer, S., Hareng, L., Hartung, T., Ahr, H., et al. (2006). The practical application of three validated in vitro embryotoxicity tests. *ATLA-NOTTINGHAM-*, 34(5), 527.
- Stephens, T. D., Bunde, C. J., & Fillmore, B. J. (2000). Mechanism of action in thalidomide teratogenesis. *Biochemical Pharmacology*, 59(12), 1489-1499.
- Therapontos, C., Erskine, L., Gardner, E. R., Figg, W. D., & Vargesson, N. (2009). 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), 8573-8578.
doi:10.1073/pnas.0901505106 [doi]
- Tijssen, H., Baars, L., & Vervoort-Peters, H. (1988). Vitamin K 2, 3-epoxide reductase: the basis for stereoselectivity of 4-hydroxycoumarin anticoagulant activity. *British Journal of Pharmacology*, 95(3), 675-682.

- Tickle, C. (2005). Making digit patterns in the vertebrate limb. *Nature Reviews Molecular Cell Biology*, 7(1), 45-53.
- Tsuiki, H., & Kishi, K. (1999). Retinoid-induced limb defects 1: inhibition of cell proliferation in distal mesenchyme of limb buds in rats. *Reproductive Toxicology*, 13(2), 103-111.
- Van Driel, D., Wesseling, J., Rosendaal, F., Odink, R., Van der Veer, E., Gerver, W., et al. (2000). Growth until puberty after in utero exposure to coumarins. *American Journal of Medical Genetics*, 95(5), 438-443.
- Vargesson, N. (2009). Thalidomide-induced limb defects: resolving a 50-year-old puzzle. *Bioessays*, 31(12), 1327-1336.
- Wilkie, A. O. (2003). Why study human limb malformations? *Journal of Anatomy*, 202(1), 27-35.
- Wiltse, J. (2005). Mode of action: inhibition of histone deacetylase, altering WNT-dependent gene expression, and regulation of beta-catenin-developmental effects of valproic acid. *CRC Critical Reviews in Toxicology*, 35(8-9), 727-738.
- Wolpert, L., Jessel, T., Lawrence, P., Meyerowitz, E., Robertson, E., & Smith, J. (2007). Organogenesis. *Principles of Development* (3rd ed., pp. 339-339-386). New York: Oxford University Press.
- Yagami, K., Suh, J. Y., Enomoto-Iwamoto, M., Koyama, E., Abrams, W. R., Shapiro, I. M., et al. (1999). Matrix GLA protein is a developmental regulator of chondrocyte mineralization and, when constitutively expressed, blocks endochondral and intramembranous ossification in the limb. *The Journal of Cell Biology*, 147(5), 1097-1108.
- Zeller, R. (2010). The temporal dynamics of vertebrate limb development, teratogenesis and evolution. *Current Opinion in Genetics & Development*, 20(4), 384-390.

Zeller, R., López-Ríos, J., & Zuniga, A. (2009). Vertebrate limb bud development: moving towards integrative analysis of organogenesis. *Nature Reviews Genetics*, *10*(12), 845-858.

Zuniga, A., Zeller, R., & Probst, S. (2012). The molecular basis of human congenital limb malformations. *Wiley Interdisciplinary Reviews: Developmental Biology*, *1*(6), 803-822.

Zur Nieden, N., Kempka, G., & Ahr, H. (2004). Molecular multiple endpoint embryonic stem cell test—a possible approach to test for the teratogenic potential of compounds. *Toxicology and Applied Pharmacology*, *194*(3), 257-269.