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# *Hox* Genes and Teratogenic Factors

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## 1. Introduction

Exposure to a variety of chemicals is a hazard of daily life. Some of these chemicals have teratogenic potency and may lead to social problems. As an example, thalidomide was prescribed as a sedative and for morning sickness in the late 1950s and caused serious embryonic effects worldwide. To avoid these problems, the development of efficient techniques for the detection of teratogenic substances contained in various chemical compounds is desired. Detection of teratogenic effects using various experimental animals is only partially effective because different phenotypes occur among species. Therefore, effective methods for the detection of teratogenic factors must be based on their molecular mechanisms. However, knowledge of the molecular mechanisms that lead to the different phenotypes caused by teratogenic factors is limited, and useful molecular markers for these factors are not known.

Approximately 30,000 genes in higher organisms are expressed under strict control. This regulation of expression is mainly dependent on transcription factor networks. In higher organisms, there are about five hundred transcription factors that contain a DNA-binding domain and cooperate in the regulation of the expression of downstream genes. Alterations in these regulatory mechanisms result in a variety of problems. In the case of teratogenic factors, abnormal morphogenesis is one of the common findings in exposed embryos. Developmental abnormalities including skeletal malformations, cleft palates, neural tube defects, and cardiovascular anomalies have been found to have a similar causative mechanism, which was revealed in loss- or gain-of-function studies of the transcription factors involved in morphogenesis. The information from genetic analyses is important for the understanding of the molecular mechanism of teratogenic effects.

The present chapter discusses the transcription factors involved in morphogenesis (*Hox*, T-box family, and other homeo-box genes) and the deleterious agents that lead to congenital malformations, and the link between them is explored. We also present recent findings from our group and provide guidelines for the prevention of the risks associated with environmental contaminants. In addition, we speculate on the molecular mechanisms of congenital malformation.

## 2. Transcription factors for development

The individual cells that make up multi cellular organisms acquire a wide variety of positional information cues from body axes. This local information leads to the formation of tissues and organs and is essential for the maintenance of homeostasis. There are three fundamental axes in multi cellular organisms: anterior-posterior, dorso-ventral, and proximal-distal. Abnormalities in body axis formation caused by genetic or external factors can lead to the development of lesions. Certain transcription factors play critical roles in body axis patterning, along with a wide variety of diffusible factors such as growth factors, BMPs, sonic hedgehog.

In this section, we refer to several transcription factors involved in body axis formation.

### 2.1 *Hox* genes

### 2.1.1 Overview of *Hox* genes

*Hox* genes encode transcription factors that play important roles in the process of morphogenesis along the anterior-posterior axis of the body. In the early 1900s, Morgan and Bridges found abnormal body plan mutants such as the replacement of antennae with legs in *Drosophila melanogaster*. These morphological abnormalities may have been caused by alterations in the expression of genes that contain a characteristic sequence (homeo-box), which were described in 1970. The homeo-box sequence encodes 61 amino acids designated as the homeo-domain and composed of a helix-turn-helix. Through the activity of this homeo-domain, Hox proteins bind a core consensus sequence (5'-TAAT-3') in target genes and function as transcriptional activators or repressors. In the regulation of the expression of various downstream genes, Hox proteins function as monomers, homodimers, heterodimers, or heterotrimers with cofactors such as Meis or Pbx family proteins (Moens and Selleri, 2006). *Hox* genes are highly conserved across species. In the nematode *Caenorhabditis elegans*, there are seven *Hox* genes in chromosome III that are distributed in intervals of 3.9 Mb. In the fly *Droshophila melanogaster*, eight *Hox* genes are clustered in chromosome 3R, which is referred to as the homeotic complex (HOM-C), and they are located across long interval regions consisting of 9.5 Mb. *Hox* genes are also clustered between 100 kb regions in the mammalian genome but this cluster is tandem duplicated; there are four clusters in the mouse (chromosomes 7, 17, 12, and 2) and humans (chromosomes 6, 11, 15, and 2). These separate clusters are termed Hox A, B, C, and D, respectively.

In normal vertebrate development, there are three important features of *Hox* gene expression. First, the genomic locations reflect the expression in the A-P axis. Generally, 3' genes are expressed in anterior tissues and 5' genes in posterior tissues. This phenomenon is termed "spatial colinearity". Second, 5' located *Hox* genes will have a dominant phenotype to more 3' located *Hox* genes. This is referred to as "posterior prevalence". The third feature is "temporal colinearity"; 3' located *Hox* genes in the cluster are expressed earlier than 5' located *Hox* genes (Mallo et al., 2010). These properties are under strict expressional control; actually a wide range of factors are involved in the control of *Hox* gene expression. A common mechanism of regulation of *Hox* expression is epigenetic control. In general, the silencing of genes is mediated by histone modifications such as the methylation of the promoter region. Polycomb and trithorax group proteins are important modulators of

histone trimethylation. The polycomb group and trithorax complexes trimethylate lysine 27 of histone H3 (H3K27) and lysine 4 of histone H3 (H3K4), respectively (Mendenhall and Bernstein, 2008). These histone modifications can be reflected in gene expression states such as inactive in H3K27m3 or active in H3K4m3. In undifferentiated pluripotent cells, two modifications are found in some local regions and are described as a bivalent chromatin domain (Bernstein et al., 2006). These chromatin modifications lead to changes in the accessibility of trans-acting factors that bind to cis-elements. The expression of *Hox* genes is regulated by a wide variety of trans-acting factors: Hox (self-, palalogus-, and another family gene) and other types of transcription factors (Cdx, Rar/Rxr, etc: see below).

Second, small or large RNAs that are independent of protein synthesis regulate *Hox* gene expression. In the *Hox* cluster, there are three miRNA families, namely mir-10, mir-196, and mir-615. These miRNAs are conserved between the fly and humans. Generally, miRNAs have been thought to influence the target mRNA stability and translation. In mammalians, at least 30 of the 39 *Hox* 3' UTRs have one or more conserved matches to miRNAs like mir-196. The expression of these mir-10 and mir-196 families is complementary to *Hox* gene expression (Mansfield et al., 2004) and is closely linked to posterior prevalence (Hornstein et al., 2005; Yekta et al., 2004). The number of registered miRNAs has recently reached more than 16,000 (miRBase, release 17), suggesting that other miRNAs from outside the *Hox* cluster may contribute to the regulation of *Hox* gene expression. LncRNAs (long noncoding RNAs), which range from several hundred bases to dozens of kilobases, are another type of RNA polymerase II transcribed RNA with a different function from a template for protein synthesis. *Xist*, known as a regulator of parental-specific expression of imprinting genes, is cited as one example (Augui et al., 2011). There are two lncRNAs transcribed from both sides of the *HoxA* cluster. *Hottip*, transcribed from the 5' site of *HoxA13*, and *Hotairm1*, transcribed from between *HoxA1* and *HoxA2*, are the recently reported lncRNAs that lead to conformational changes in chromatin together with the transcribed *Hox* gene RNA (Rinn et al., 2007; Wang et al., 2011).

The regulation of the expression of the *Hox* cluster genes occurs through a wide variety of mechanisms, and irregularities in this regulation can result in several abnormalities as described in the following sections.

### 2.1.2 Phenotypes of *Hox* mutants

Loss or gain of function *Hox* mutants show homeotic transformations across species. The identity of body segments is determined by specific combinations of *Hox* gene expression known as the "Hox-code". Alterations in the Hox-code result in abnormalities in morphogenesis along the longitudinal (A-P) axis of the body, termed homeotic transformation. For example, loss of function of the *labial* gene in the fly, which is located in the 3' region of the cluster, results in the disorganization of cranial structure, which is seen in the formation of the salivary glands. The *antennapedia* mutant is characterized by the replacement of antennae by legs (Hughes and Kaufman, 2002). The HOM-C complex is formed by two gene clusters, the *antennapedia* complex (*ANT-C*) and the *bithorax* complex (*BX-C*). These two complexes are encoded in the same chromosome but are separated by 9.5 Mb. Genetic analyses have shown that *ANT-C* determines the specificity of the anterior thoracic and head regions, and *BX-C* determines the posterior thoracic segments and the abdomen.

In higher organisms, there are 39 known *Hox* genes and the analysis of their function has become increasingly more complex. The analysis of the function of mammalian *Hox* genes in vivo has been carried out through the generation of a large number of *Hox* gene knockout or knock-in mice. These mice frequently show skeletal abnormalities such as alterations in the number of thoracic segments. These phenotypes are explained as resulting from aberrances in the Hox-code (Wellik, 2009). Dramatic phenotypes, such as the replacement of antennae by legs in the fly mutant, are not observed in single gene mutants in mammals possibly because of compensatory effects between the 39 *Hox* genes, especially among paralogous genes.

Individual *Hox* genes have specific functions in various organs. Among skeletal abnormalities, cleft palate phenotypes have been detected in *Hoxa2⁻/⁻* mice (Gendron-Maguire et al., 1993). These phenotypes have also been observed in *Hoxa7* and *Hoxb7* gain of function mice (Balling et al., 1989; McLain et al., 1992).

The expression of *Hox* genes belonging to paralog groups 9 to 13 are coordinately detected during limb bud development. Among these genes, *Hoxa13* and *Hoxd13* in the paralog group 13 are specifically expressed in the developing distal region (the autopod). Human synpolydactyly (SPD) is a rare dominantly inherited limb malformation characterized by syndactyly between the third and fourth fingers and between the fourth and fifth toes. Typical SPD is caused by mutations of the *Hoxd13* gene such as expansions, frame-shift deletions, and functional mutations (Malik and Grzeschik, 2008). Another human malformation, the hand-foot-genital syndrome (HFGS), is associated with mutations in the *Hoxa13* gene (Goodman, 2002).

In addition, aberrant limb formation has been observed in *Hoxa13⁺/⁻* and *Hoxd13⁻/⁻* mutant mice (Dolle et al., 1993; Fromental-Ramain et al., 1996). The greater severity of the phenotypes of *Hoxa13⁺/⁻/Hoxd13⁻/⁻* mice suggested that redundancies within paralog groups may play a role in limb development. These redundant manners are also observed in kidney formation. There are three *Hox11* paralogous genes in clusters A, C, and D. In triple mutants, metanephric induction is completely absent, and the reduction of *Six2* and *Gdnf* expression is believed to be one of the causative factors for this phenotype (Wellik et al., 2002). On the other hand, the activities of *Hox* genes within a single cluster are important for kidney formation (Di-Poi et al., 2007).

*Hoxa13⁻/⁻* and *Hoxd13⁻/⁻* mice exhibit a reduction of branching in prostate ducts; *Hoxa13⁻/⁻* mutants die *in utero* with severe urinary and genital tract malformations (Podlasek et al., 1999; Podlasek et al., 1997; Warot et al., 1997). *Hoxb13* also functions in ventral prostate morphogenesis. *Hoxb13⁻/⁻* mice exhibit transparent ducts of the ventral prostate and these abnormalities are more severe in *Hoxb13⁻/⁻/Hoxd13⁻/⁻* mice, which show a 50% reduction in the number of duct tips (Economides and Capecchi, 2003).

The involvement of the *Hox* genes described above in various morphogenetic events suggests the possible existence of a close relationship between *Hox* gene expression and the effects of teratogenic factors.

### 2.1.3 Function of *Hox* genes: Proliferation, apoptosis, and differentiation

*Hox* genes have different functions associated with proliferation, apoptosis, and differentiation. The *Hox* genes of the A, B, and C but not of the D clusters are transcribed in specific subpopulations during normal hematopoiesis. Gain- or loss-of-function analyses of

expressed *Hox* genes showed their ability to specifically regulate different stages of hematopoietic development. Among them, *Hoxb4* serves as a positive regulator of self-renewal and expansion (engraftment) in hematopoietic stem cells (HSC) (Antonchuk et al., 2002; Kyba et al., 2002). On the other hand, definitive hematopoiesis was not disrupted in *Hoxb4*-deficient mice, but *Hoxb3−/−/Hoxb4−/−* mice exhibited more pronounced hematopoietic differences (Bjornsson et al., 2003; Sauvageau et al., 1995). These results suggest that a more complex mechanism, such as gene redundancy, compensatory mechanisms and cross-regulatory interactions, among *Hox* genes may play a significant role in vivo.

*Hox* genes are involved in the regulation of cell proliferation, and their expression in tumor cells has therefore been studied. Certain *Hox* genes show aberrant expression in various tumor cells (Shah and Sukumar, 2010). These disruptions of normal *Hox* expression may affect various pathways linked to the promotion of tumorigenesis and metastasis. Moreover, some *Hox* genes (*Hoxa9, 11*, and *13*) are fusion partners of the nucleoporin gene *Nup98* in human leukemia (Moore et al., 2007). This fusion oncoprotein may play a role in modulating transcription and controls the nucleo-cytoplasmic transport of some mRNAs and proteins. Actually, the *Nup98-hox* fusion induces myeloproliferative disease and AML in mouse bone marrow transplantation models. Other fusion proteins such as *Hoxc11* or *13* are also known to induce AML.

Tumorigenesis is often characterized by alterations in the balance between proliferation and apoptosis. When viewed from this perspective, alterations in *Hox* gene expression can contribute to tumorigenesis (oncogenesis) by allowing the activation or repression of the apoptosis pathway. In breast cancer cell lines, Hoxa5 directly regulates *p53* expression by binding to its promoter (Raman et al., 2000). In addition, Hoxa5 induces apoptosis by promoting the expression of caspase 2 and caspase 8 in breast cancer cells in a p53-independent manner (Chen et al., 2004). In the fly, *Hox* genes induce the localized cell death that is essential for the maintenance of a morphological boundary between the two structures of the embryo's head, namely the maxillary and mandibular head lobes. In this case, the *Hox* gene *Deformed (Dfd)* directly activates the expression of the cell death promoting gene *reaper (rpr)*, thereby inducing localized cell death (Lohmann et al., 2002). Although it is not clear whether this apoptotic pathway is conserved in mammalian cells, some *Hox* genes show a close relationship to the apoptosis pathway.

*Hox* genes play crucial roles in differentiation. In HSC, the expression of *Hox* genes is downregulated during differentiation and maturation. The gain or loss of some *Hox* genes causes alterations in hematopoietic lineage commitment (He et al., 2011). Neural crest cells (NCCs) are also multipotent and can differentiate into different cell types, including peripheral and enteric neurons, glia, melanocytes, and smooth muscle. The *Hox* genes specify the location of the NCCs and contribute to the differentiation process (Minoux and Rijli, 2010).

Recent observations indicated that some *Hox* genes also have multiple functions in higher order biological mechanisms. Grooming is a stereotypic behavior in mammals and energizes the various regions of the brain, such the brainstem, striatum, and cortex. Excessive grooming manifests itself in humans as the obsessive-compulsive spectrum disorder trichtilomania. *Hoxb8* knockout mice show the excessive grooming phenotype. This abnormal behavior becomes a cause of death in knockout mice (Greer and Capecchi, 2002). Cell lineage tracing showed that this aberrant behavior can be attributed to the lack of bone

marrow-derived microglia cells (Chen et al., 2010). The reduction of the total number of microglia cells in the adult brain of *Hoxb8* mutants is clear, although it remains unknown why the disruption of *Hoxb8* function only affects a small fraction of microglia cells and whether *Hoxb8* promotes the proliferation, differentiation or activation of apoptosis in a subpopulation of microglia cells.

These results indicate *Hox* genes are involved in a wide range of biological functions. However, the role of these genes in various processes is not entirely clear and knowledge of the direct targets of *Hox* genes is quite limited. We identified Hox protein target genes using chromatin immunopurification (ChIP) (Tomotsune et al., 1993). The use of modified methods, such as ChIP-sequence, is necessary to obtain further information on target genes and to understand their mechanisms of action.

## 2.2 *Cdx* family

The *Hox* cluster is believed to have arisen through the duplication of an ancestral ProtoHox cluster in early metazoan evolution. In this process, the ParaHox cluster genes, which show close evolutionary relationships, also arose (Garcia-Fernandez, 2005). *Cdx* (caudal-type homeo-box) genes are ParaHox genes, and three paralogous *Cdx* genes are present in the mouse genome and are located in different chromosomes. *Cdx* genes are required to correctly pattern the head to tail axis.

In *Cdx1$^{-/-}$* mutants, an anterior homeotic transformation involving the occiput and the first three cervical vertebrae is observed. These changes are accompanied by a posterior shift of *Hox* expression involving three different clusters (Subramanian et al., 1995).

*Cdx2$^{-/-}$* mutants present a much more severe phenotype and die between E3.5 and E5.5. The embryonic lethality of these mutants may be attributed to aberrations in the maturation of trophoblasts from the trophoectoderm. *Cdx2$^{+/-}$* animals are viable but show tail abnormalities and growth retardation. Anterior homeotic transformation in the lower cervical and upper thoracic regions is also observed in skeletal analyses (Chawengsaksophak et al., 1997).

*Cdx4* is an X-linked gene and no significant abnormality is observed in either sex in *Cdx4$^{-/-}$* embryos (van Nes et al., 2006). However, double knockout *Cdx1$^{-/-}$/Cdx4$^{-/0}$* mice were reported to show alterations in skeletal structure, and *Cdx2$^{+/-}$/Cdx4$^{-/0}$* embryos were lethal around E10.5, which was attributed to a developmental defect of the chorio-allantoic placenta.

*Cdx2$^{+/-}$/Cdx1$^{-/-}$* mutants show a greater degree of posterior truncation (van den Akker et al., 2002). These results indicate the existence of redundancy in the *Hox* clusters among the *Cdx* family genes. The *Cdx* family genes directly regulate some *Hox* genes, such as *Hoxa5* and *Hoxb8* (Subramanian et al., 1995; Tabaries et al., 2005), as these *Hox* genes were shown to rescue the *Cdx* mutant phenotypes (Young et al., 2009).

## 2.3 Another ParaHox gene

*Hox* and ParaHox genes belong to the ANTP class of homeobox genes. The ANTP class also includes two other genes, *Evx* (even-skipped homeotic) and *Meox* (mesenchyme homeobox). These two ParaHox genes, *Evx* and *Meox,* are located on either side of the *Hox* cluster in

vertebrates. In the mouse genome, *Evx* and *Meox* each have two paralogous genes (*Evx1*, *Evx2, and Meox1, Meox2*) in different chromosomes. *Evx1⁻/⁻* is characterized by early embryonic lethality as it fails to differentiate extraembryonic tissues or to form egg cylinders (Spyropoulos and Capecchi, 1994). *Evx2⁻/⁻* mutants show malformation of the autopod, and these results indicate that *Evx2* has a genetic interaction with *Hoxd13* (Herault et al., 1996). *Meox1⁻/⁻* mutants exhibit hemi-vertebrae and rib, vertebral and cranial-vertebral fusions (Jukkola et al., 2005). *Meox2⁻/⁻* mutants show mild defects of rib and vertebral development (Mankoo et al., 1999).

*NK* (Nirenberg and Kim) homeo-box genes are evolutionary relatives of both *Hox* and ParaHox genes. In the mouse genome, there are ten *Nkx* family genes that are located in seven different chromosomes. *Nkx* family genes are mostly observed in mesodermal derivatives; in particular, *Nkx2.5* is essential for cardiac muscle differentiation (Hatcher et al., 2003).

## 2.4 T-box family

The T-box family genes encode a common DNA-binding domain known as the T-box and are also evolutionarily conserved transcription factors. This family of genes is composed of two independent functional domains: the T-box in the large N-terminal region and a transcriptional activation/repression domain in the C-terminal region. The *Brachyury* (or *T*) mouse mutant is characterized by a truncated tail and was discovered about 80 years ago. Until recently, 17 genes were identified as T-box family genes in vertebrates, and genetic analyses of individual genes have progressed significantly. These analyses indicate that T-box genes are widely involved in developmental processes of mesoderm specification (Naiche et al., 2005). In humans, mutations in T-box genes, including deletions, rearrangements, missense mutations, insertions, and truncation, lead to various genetic disorders (Packham and Brook, 2003). The phenotypes of T-box gene knockout mice can be compared with the phenotypes of several human genetic disorders.

*Tbx1⁻/⁻* mice have a lethal phenotype in late gestation and display a wide range of developmental abnormalities including facial abnormalities, cleft palate, cardiac outflow tract defects, and hypoplasia of the thymus and parathyroid glands (Jerome and Papaioannou, 2001; Merscher et al., 2001). In humans, chromosome 22q11 deletion syndrome is known as DiGeorge and velocardiofacial syndrome (DGS/VCFS) and its phenotypic characters include anomalies of the cardiac outflow tract, cleft palate, facial dysmophogenesis, and hypoplasia of the thymus and parathyroid glands. *Tbx1* is located in a region spanning 3 Mb, but another genes are also located in this region. *Tbx1* is thought to be a key gene in the etiology of DES/VCFS.

*Tbx3⁻/⁻* mice show a deficiency of mammary gland induction, genital abnormalities, and forelimb and hind limb malformations, and die during gestation(Davenport et al., 2003). However, *Tbx3⁺/⁻* mice appear fairly unaffected. Ulnar-mammary syndrome (UMS) is a pleiotropic disease associated with malformations of the posterior elements of the upper limbs, apocrine/mammary hypoplasia and/or dysfunction, dental abnormalities, and genital anomalies. Clinical manifestations are highly variable. Many similarities are exhibited in the phenotype, but gene dose sensitivities are different between humans and mice.

*Tbx4+/-* mice form hind limb buds; however, they fail to outgrow them. *Tbx4-/-* mutants have problems with the allantoic connection to the placenta and die early in embryogenesis (Naiche and Papaioannou, 2003). The mutations in the human *Tbx4* gene are linked to an autosomal dominant disorder called small patella syndrome (SPS) (Bongers et al., 2004).

*Tbx5-/-* resulted in early embryonic lethality due to severe defects in early heart formation (Bruneau et al., 2001). Holt-Oram syndrome is an autosomal dominant disorder that includes cardiac and upper limb malformations. *Tbx5+/-* mutants faithfully recapitulate the human phenotype, including cardiac defects and forelimb malformations.

As *Tbx19* (known as *Tpit*) expression is restricted to the pituitary gland, *Tbx19-/-* mutants show a significant reduction in the number of pituitary POMC (pro-opiomelanocortin)-expressing cells (Pulichino et al., 2003). In humans, the absence of pituitary POMC leads to a lack of adrenocorticotrophin (ACTH), resulting in adrenal insufficiency.

The *Tbx22-/-* mutation caused death within 24 hr after birth due to submucous cleft palate and ankyloglossia (Pauws et al., 2009). In humans, *Tbx22* is a gene responsible for X-linked cleft palate and ankyloglossia (Braybrook et al., 2001).

## 3. Developmental toxicants

Teratology is the study of abnormal development and congenital malformations attributed to genetic factors, maternal factors, toxicants, or other factors such as environmental chemicals. In the early 1970s, James G. Wilson created "The Six Principles of Teratology". These principles are still applied today and guide the investigation of teratogenic agents and their effects on the development of organisms. A wide variety of chemicals and environmental factors are believed to have teratogenic potential in humans and animals. In the USA, the Food and Drug Administration (FDA) has categorized drugs into five different risk categories for pregnant women. These five categories (A, B, C, D, and X) have been considered a therapeutic advantage but they are only based on specific criteria and are not universal. Therefore, the number of these factors is likely higher than one thousand and is increasing daily. The teratogenic potentials of various chemicals and environmental factors are determined using animal models (e.g., zebra fish, mouse, rat, pig, rabbit, dog, and monkey). In the past, observations of the characteristics of embryos from candidate-exposed pregnant animals were used as the main criteria, but current teratological evaluations need to include knowledge about the molecular mechanisms.

In this section, six xenobiotics known to be developmental toxicants in humans were selected for further description.

### 3.1 Endocrine disruptors

Under normal conditions, hormones are involved in the maintenance of homeostasis, but endocrine disruptors, which are environmental chemicals that have a hormone action or inhibit the activity of an endogenous hormone, have an adverse effect on organs and progeny. In adults, the role of the endocrine system in the maintenance of homeostasis is established and adults therefore have resistance against endocrine disruptors. However, in the fetus, infants, and children, resistance against these agents may be weak and they therefore can have irreversible impact on developmental functions such as organ formation.

Endocrine disruptors are found in low doses in products of daily use. DTT, polychlorinated biphenyls (PCBs), bisphenol A (BPA), polybrominated diphenyl esters (PBDEs), and a variety of phthalates are chemicals used in pesticides, plastic food containers or plastic toys, and are currently recognized as endocrine disruptors.

Methoxychlor (MXC) is an organochlorine DDT derivative. MXC was shown to affect fertility in mice and cause maternal weight gain in fetal rats. These effects are thought to be mediated by the inhibition of estrogen binding to the Estrogen Receptor (Esr), by MXC and the suppression of the expression of *Hoxa10* (Fei et al., 2005).

BPA also affects estrogen signaling. In BPA-exposed males, an increase in the size of the prostate gland and oligospermia are observed. BPA exposure negatively regulates the expression of *Hoxa10* and *Hoxa11* and affects estrogen signaling (Varayoud et al., 2008).

Estrogen and androgen are sex steroids that are needed for the proper development of reproductive organs (Dupont et al., 2000). Each steroid binds to a specific nuclear-receptor and these receptors act as ligand-dependent transcription factors. Alterations in *Hox* gene expression induced by endocrine disruptors is thus considered to be a nuclear receptor-mediated mechanism.

### 3.2 Diethylstilbestrol (DES)

DES is a synthetic nonsteroidal estrogen. This chemical was used as a pharmaceutical product for the prevention of miscarriage in the 1970s, but vaginal cancer occurred frequently in children whose mothers took DES. Exposed offspring also experienced a high incidence of pregnancy wastage and preterm labor. DES induced altered *Hox* gene expression in human uterine endometrial and cervical cells. In reproductive organs, *Hox* genes are also expressed along the A-P axis. Under this rule, *Hoxa9* is normally expressed in the oviduct and *Hoxa10* in the uterus. However, this linear regulation of expression is disrupted in DES-exposed humans or mice, and the expressional domain is shifted posteriorly (Block et al., 2000). DES binds the Esr, and the irregular nuclear receptor activation may lead to aberrant *Hox* gene expression.

### 3.3 Anticonvulsants (VPA)

Valproic acid (VPA) is a chemical compound used for the treatment of epilepsy. However, VPA has teratogenic potential along with other anticonvulsant drugs, including carbamazepine and phenytoin in humans. Having weak developmental toxicant of carbamazepine and phenytoin, VPA exposure is more toxicant. In humans, infants exposed to VPA *in utero* show anomalies including neural, craniofacial, cardiovascular, and skeletal defects. A similar teratology is exhibited in rodents, rabbits, and nonhuman primates. The spina bifida, a neural tube defect, is also observed in VPA-exposed infants at frequencies of 1–2%. VPA exposed human embryonic carcinoma (NTera2/D1) cells show slight alterations in the expression of certain *Hox* genes (Faiella et al., 2000). VPA is also an inhibitor of class I and IIa HDACs (histone deacetylases); therefore, changes in the expressions of various genes are thought to occur in different tissues. However, it remains largely unknown why phenotypes appear only in limited organs in which HDAC is the primary target of VPA, and the mechanisms underlying the action of VPA are not clear.

### 3.4 Thalidomide

Thalidomide (α-phthaliidoglutarimide) was used as a sedative and for morning sickness in the late 1950s. However, reports of the teratogenic potency of thalidomide appeared in the early 1960s. In these reports, infants exposed to thalidomide during the early stages of pregnancy had multiple defects, such as malformations of the limbs, ears, eyes, internal organs and central nervous system. The most commonly observed defects were limb malformations including amelia (complete absence of the limb) and phocomelia (truncation or absence of the zeugopod). Thalidomide-induced limb defects are observed in humans, monkeys, rabbits and chicks, but these phenotypes are not observed in the mouse or rat (Vargesson, 2009). For 50 years, the mechanism of thalidomide-teratogenicity was poorly understood. Thalidomide is a derivative of glutamic acid and contains two imide rings: glutarimide and phthalimide. Thalidomide has therefore been believed to act by causing biochemical alterations of glutamic acid, nucleic acids and vitamin B. Recently, the primary target of thalidomide was identified and parts of the molecular mechanism were revealed (Ito et al., 2010). A thalidomide-binding protein, *Cereblon* (CRBN), forms an E3 ubiquitin ligase complex with Ddb1 and Cul4A in a thalidomide-dependent manner and modifies the expression of *Fgf8* in the limb. Generally, the limb has three developmental axes: the proximal-distal axis, which runs from the base of the limb to the tip; the A-P axis, which runs parallel with the body axis; and the D-V axis, which runs from the back of the hand to the palm. Under the control of these three axes, various positional identities are specified by the concentration gradient of diffusible factors, such as *Fgfs*, *Bmps*, *Wnt*, and *Sonic Hedgehog*. The malformed fin or limb in knockdown *Crbn* zebrafish or the dominant negative form of *Crbn* expressing chicken, respectively, indicated the important role of the ubiquitin ligase pathway for morphogenesis through thalidomide, and clearly showed that the reduction of *Fgf8* expression lead to the deformity of the limb. Although there is no information about the specific target molecule of *Crbn*, identification of this target molecule will allow a more effective use of thalidomide for the treatment of multiple myeloma and erythema nodosum leprosum and avoid its associated teratogenic risks.

### 3.5 Retinoic acid

In normal embryogenesis, a wide variety of diffusible factors act as morphogens and control proper morphogenesis. Retinoic acid is one of the morphogens that function during the formation of various organs such as the head, trunk, limbs, heart, and the central nervous system. Retinol and other retinoid compounds, which are precursors of retinoic acid, cannot be synthesized *de novo* and must therefore be ingested in food or supplements. After modification of ingested retinol by multiple enzymes, the resulting compounds bind to ligand-activated nuclear receptors, namely Rar (retinoic acid receptor) and Rxr (retinoid X receptor). *Rar* and *Rxr* are concurrently encoded by three family genes (α, β, and γ), and subtypes exist that are products of alternative splicing or different promoter usage. The activated nuclear receptors form homo- or hetero-dimers and recruit coactivators or corepressors to the RARE (retinoic acid response element) on the target genes. Some target genes have been identified and these genes function in the cell cycle, proliferation, and morphogenesis (Delacroix et al., 2010; Nielsen et al., 2008).

Retinoids were found to be teratogenic in humans in the early 1980s. Infants exposed during gestation have teratogenic syndrome including craniofacial abnormalities and defects of the

thymic, cardiovascular, and central nervous system. The teratogenic effect is observed in other animal models, such as mouse, rat, pig, rabbit, dog, chick, and monkey. The dosage and timing of gestational exposure profoundly influence the form of the birth defect and the lethality, but differences in the genetic background in mice also have an effect. The peak period of sensitivity for a given tissue appears to be during the development of the primordial structures.

*Rar* and *Rxr* knockout mice were produced by several methods and these mice exhibited various phenotypes (Mark et al., 2006). However, their abnormalities were restricted to a subset of tissues normally expressing these receptors, probably reflecting the existence of functional redundancies between nuclear receptors. The expression of certain *Hox* genes is controlled via RARE (Oosterveen et al., 2003). Although differences in the RA exposure dosage determine the phenotypes, aberrant *Hox* gene expression is thought to play a role in the morphological malformations.

## 3.6 Dioxin

PCDDs (polychlorinated dibenzo-p-dioxins), PCDFs (polychlorinated dibenzofurans) and DL-PCBs (dioxin-like polychlorinated biphenyls) are generically termed as dioxin and are similar toxicants. Dioxin is an environmental contaminant and is unintentionally generated as a by-product of industrial combustion. Among dioxins, TCDD (2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin) is the most severe toxicant and has the highest teratogenic potency in mammals. Differences in the exposure dosage determine the resulting phenotypes, which include cleft palate, hydronephrosis, and defects of sex organs. There are differences due to timing and dose of exposure, TCDD exposure causes a significant decrease in ventral prostate development, which is similar to the effect of impairment of certain *Hox* genes (Vezina et al., 2009), but the correlativity between TCDD and *Hox* expression is not cleared.

The molecular mechanisms of the xenobiotic dioxin pathway can be understood through the analyses of nuclear receptors. The toxicant and teratogenic effects of dioxin are thought to depend on the activation of the Ahr (aryl hydorocarbon receptor) pathway. Ahr has multiple functions. Ahr is a ligand-activated transcription factor that is a member of the bHLH/PAS (basic Helix-loop-Helix/Per-Arnt-Sim) family of genes. Ahr generally localizes to the cytoplasm and forms a complex with Hsp90 (heat shock protein 90), cochaperone p23, and the immunophilin-like protein Ara9 (Bell and Poland, 2000). Upon binding ligand, Ahr is released from the p23/Ara9 complex and translocates to the nucleus. In the nucleus, Ahr dissociates from Hsp90 and dimerizes with another bHLH-PAS family gene, Arnt (Ahr nuclear translocator). The Ahr/Arnt heterodimer binds to specific DNA sequences termed XREs (xenobiotic response elements: 5'-GCGTG-3') and regulates target gene expression with transcriptional coactivators/corepressors, which are also partners of Rar/Rxr (Beischlag et al., 2002). Currently, various genes are reported as Ahr target genes: phase I drug metabolizing enzymes, such as those of the *Cyp450* (Cytochorome 450) family; phase II enzymes, such as *Ugt1a1* (Kohle and Bock, 2007); and as down stream genes of Ahr-pathway, such as cytokines, and *Tgf* family which are unrelated xenobiotic metabolism genes (Haarmann-Stemmann et al., 2009).

Ahr is also a modulator of estrogen receptor signaling. Ohtake et al. indicated that the ligand-activated Ahr/Arnt heterodimer directly associates with the Esr, recruits a coactivator, and regulates the expression of Esr target genes (Ohtake et al., 2003). In this

case, Ahr functions as activator/repressor depending on the ligand binding state of the Esr. Another function of ligand-activated Ahr as a substrate-specific adaptor in the Cullin 4B ubiquitin ligase complex was described (Ohtake et al., 2007). In this case, agonist (3MC: 3-methylcholanthrene)-activated Ahr ubiquitinates Esr1 and androgen receptor (Ar) in vitro and in vivo. In *Ahr*-deficient mice, responses to dioxin such as the induction of *Cyp1a1* activation were not observed. However, null mutants have functional impairments of male and female reproductive organs, depending on the affected allele (Schmidt et al., 1996). Although there are other identified ligands for Ahr, including flavonoids, UV photo-products of tryptophan and some synthetic retinoids, the above observations suggest that Ahr plays a role in the cross talk between the dioxin and RA pathways.

## 4. Hox-RA/dioxin

The identification of molecular markers for the variety of existing teratogenic factors is an urgent need in various respects. In the present report, we focused on the expressional changes of the *Hox* cluster genes in embryos exposed to teratogenic factors because their aberrant expression lead to various morphological defects and the affected animals have close similarities (Kojima and Takahashi, 2009).

Because the different amount and timing of exposure to teratogenic factors are correlated to the different effects in embryos, we firstly examined the RA or dioxin effects in E10.5 embryos for 6 hr by one-shot administration. This dose induced craniofacial and skeletal defects in RA-exposed embryos, and hydronephrosis in dioxin-exposed embryos. Among 39 *Hox* cluster genes, 3′-located paralogs (*Hox1~8*) were up-regulated and some 5′-located genes (*Hoxa11, Hoxd9, and Hoxd12*) were down-regulated in the RA-exposed embryos (*Fig. 1*). Meanwhile, the influence of relative position in the cluster was not observed in the TCDD-exposed embryos. A and D cluster genes were down-regulated and no clear difference was observed in the cluster B. Additionally, aberrant expression of pri-miRNAs (precursors of miRNAs) was detected (*Fig. 2*). These pri-miRNA changes were correlated with changes in the expression of closely located *Hox* genes.

TCDD exposure causes a decrease in the levels of all-trans RA in the liver, which is the main RA storage location in a variety of species (Fletcher et al., 2001). There are some similarities in terms of morphological changes between RA- and TCDD-exposed embryos. The developmental effects of the replacement of RA storage by TCDD are not clear, but it is suggested that TCDD and related compounds have an impact on retinoid homeostasis and the RA signaling pathway. Our analyses indicate that alterations in the expression of *Hox* cluster genes do not show a clear correlation with these teratogenic factors. In addition, expressional changes of some pri-miRNAs in the *Hox* clusters are also different between these two factors. Although the involvement of Ahr in the cross talk between the RA and TCDD pathways is possible, there are clear differences in the downstream effects, such as expressional changes of *Hox* cluster genes. We also detected changes in the expression of other transcription factors, such as ParaHoxs and T-box family genes, in embryos exposed to these factors. Some genes show a specific response to each teratogenic factor. Based on these results, changes in the expression of transcription factors can be considered as potential molecular markers for the verification of teratogenic effects. Therefore, to better determine the teratogenic potential of various chemicals, further investigation of the effects of the timing or dosage of RA and TCDD in exposed embryos is under way.
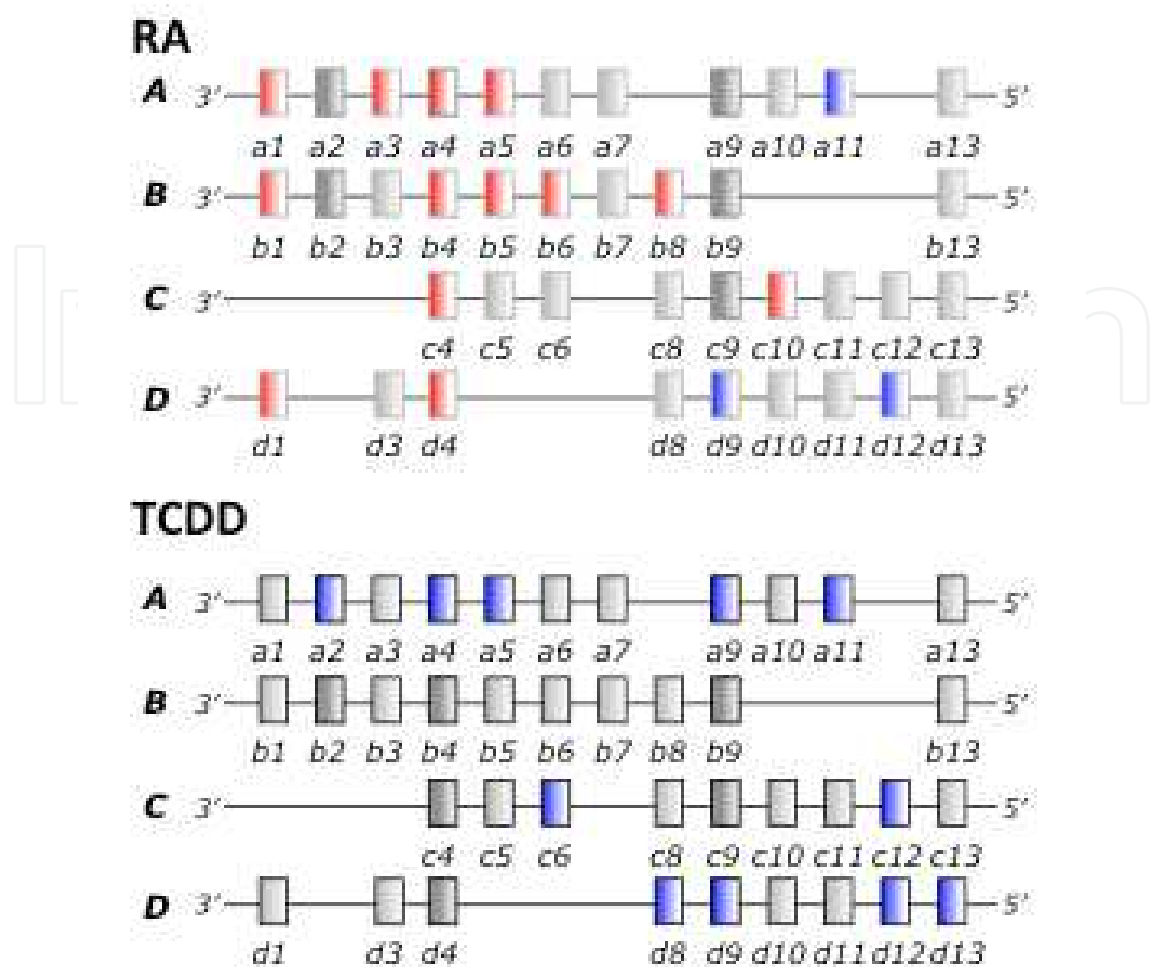
Fig. 1. Expressional changes of the *Hox* cluster genes in embryos treated with teratogenic agents. 39 *Hox* genes are separated on four clusters (A, B, C, and D) in four chromosomal loci. The box indicates each gene. Red boxes indicate up-regulated and blue boxes indicate down-regulated genes in response to treatment with teratogenic agents.
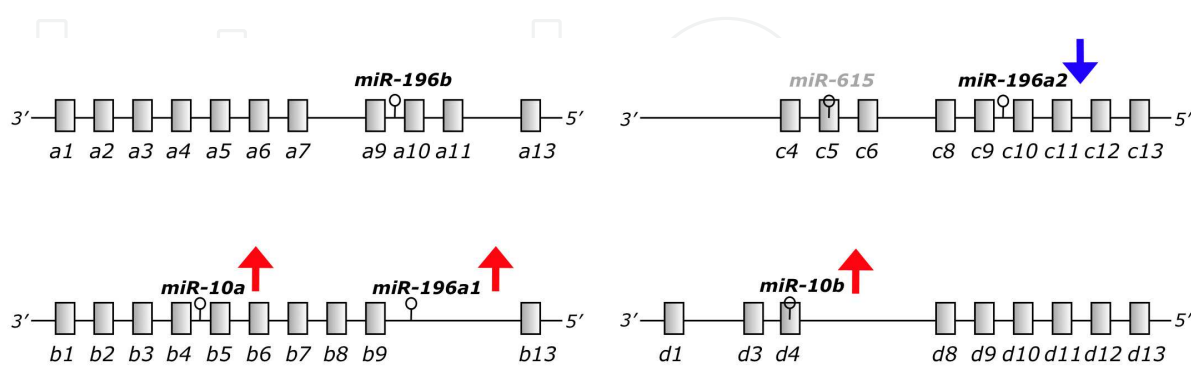


Fig. 2. Expressional changes of pri-miRNAs in the *Hox* cluster genes. Three miRNA family genes (*miR-196, miR-10, and miR-615*) are located in the *Hox* cluster. Red arrows indicate RA and the blue arrow indicates TCDD exposure. The upward direction of the arrow represents increased expression and the downward direction represents decreased expression.

## 5. Conclusions

Contrasting a comprehensive analysis using DNA microarrays, our analysis is simpler and allows the examination of a large number of samples. Information on molecular markers such as the *Hox* genes under various conditions (exposure-time, -dosage) will allow the prediction of the hazardous nature of unknown factors. In addition, the understanding of the molecular mechanisms common to different teratogenic agents requires the identification of the target genes of Hox protein and each transcription factor and an understanding of transcription factor networks.

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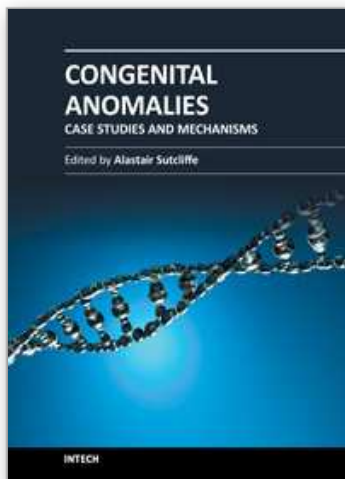
**Congenital Anomalies - Case Studies and Mechanisms**

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This book is in essence a collection of essays which are state of the art in their respective areas of knowledge. They inform the reader of all sorts of mechanistic considerations when developing understanding of issues surrounding the origins of congenital abnormalities. These chapters are written by world renown authorities in this area of science and represent a wide range of expertise from a clinician perspective, through to genetic mechanisms. Unlike some books which take a formal textual, somewhat plodding way through pathophysiology here instead we have cut through chapters in which the student , or scientist or medic is lead to understand just how complex the origins can be via examples from different parts of the body. With the erudite chapters are relevant tables and other diagrams to help clarify the text . These chapters represent a starter text for the stimulus for further knowledge of what is an increasingly important area of human health.

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